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Functional Characterisation of the Human S1P₄ G Protein-Coupled Receptor

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A thesis submitted in partial fulfilment of the
requirements of the Open University for the degree of
Doctor of Philosophy

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*“...man will occasionally stumble over the truth,
but usually manages to pick himself up, walk over
or around it, and carry on.”*

Winston S. Churchill (1874-1965)

Abstract

The human G protein-coupled receptor (GPCR) S1P₄ (formerly named Edg6) was identified as an orphan receptor and assigned to the Edg family on the basis of sequence similarity with other Edg receptors. S1P₄ was recently shown to couple to G α_i and G $\alpha_{12/13}$ G proteins in response to stimulation by the lysophospholipid, sphingosine-1-phosphate (S1P). This thesis describes functional characterisation of the S1P₄ receptor using a modified [³⁵S]GTP γ S binding assay.

CHO-K1 cells were stably transfected to express S1P₄, or a fusion protein between S1P₄ and a pertussis toxin-resistant form of G α_{i1} . S1P₄ exhibited significant constitutive activity and treatment with S1P or phytoS1P further stimulated the receptor in a dose-dependent manner. Pertussis toxin treatment demonstrated that both S1P₄ constitutive activity and the effects of S1P were transduced via endogenous G α_i G proteins, whilst the S1P₄-G α_{i1} fusion signalled via the tethered, pertussis toxin-insensitive G protein. Residue ¹²² was shown to be important in governing S1P₄ ligand selectivity; mutation of the naturally occurring glutamic acid to glutamine attenuated the ability of the receptor to respond to S1P and conferred sensitivity to the related compound, lysophosphatidic acid.

Since most cell types express endogenous S1P and LPA family receptors, the profile of Edg family receptor expression in CHO-K1 cells was investigated. Transcripts for S1P_{1,2,4} and LPA₁ receptors were detected using RT-PCR with species-specific oligonucleotides.

Further investigations into S1P₄ constitutive activity, which were undertaken using an inducible expression system, demonstrated direct correlation between S1P₄ expression and constitutive activity. This suggested that S1P₄ constitutive activity represents an inherent property of this receptor. In common with other naturally constitutively active GPCRs, S1P₄ lacks a generally conserved cysteine residue within the first extracellular loop. It is proposed that this may account for S1P₄ constitutive activity and may be relevant to the *in vivo* function of this receptor.

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I'd like to thank my friends, who showed an interest in what I was doing and wondered why I answered through gritted teeth when they asked if I'd finished yet. Anna, Sarah and Krystyna did a fantastic job of humouring me, talking shop and then reminding me that there was life beyond the lab – cheers guys. I understand the most effective means of thanking you all is by buying the beers. I'm also very grateful to my Mum for proof-reading my thesis.

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Abbreviations

5-HT _{1A}	5-hydroxytryptamine 1A receptor
5-HT _{2B}	5-hydroxytryptamine 2B receptor
Å	angstrom
A (aa)	alanine
A (nt)	adenine
aa	amino acid
AAL	2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol
α _{1A} AR	α _{1A} adrenergic receptor
α _{2A} AR	α _{2A} adrenergic receptor
ANOVA	analysis of variance
ATP	adenosine 5'-triphosphate
BCA	bicinchoninic acid
Bcl-2	B cell lymphoma 2
β ₁ AR	β ₁ adrenergic receptor
β ₂ AR	β ₂ adrenergic receptor
β-Arr	β-arrestin
BGH	bovine growth hormone
BH3	Bcl-2 homology 3 domain
bp	base pairs
Bq	Becquerel
BSA	bovine serum albumin
C	Celsius
C (aa)	cysteine
C (nt)	cytosine
CAM	constitutively active mutant
cAMP	adenosine 3',5'-cyclic monophosphate
CB	cannabinoid
CCR1	CC chemokine receptor 1
cDNA	complementary DNA
CHO	Chinese hamster ovary
Ci	Curie
CMV	cytomegalovirus
CNS	central nervous system
CRLR	calcitonin receptor-like receptor
CsA	cyclosporin A
CTR-2	calcitonin receptor 2
CXCR1	CXC chemokine receptor 1
D (aa)	aspartic acid
D2 _{long}	dopamine D2 long isoform receptor
Da	dalton
DAPI	4',6'-diamidino-2-phenylindole
DBD	DNA binding domain
DCM	dichloromethane
dhS1P	dihydrosphingosine-1-phosphate
DMEM	Dulbecco's modified Eagle's medium
DMS	dimethylsphingosine
DMSO	dimethylsulphoxide

DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNTP	deoxyribonucleoside triphosphate
dpm	disintegrations per minute
DTT	dithiothreitol
E (aa)	glutamic acid
<i>E. Coli</i>	<i>Escherichia Coli</i>
e.g.	<i>exempli gratia</i> (for example)
E/GRE	ecdysone/glucocorticoid receptor elements
EBV	Epstein-Barr virus
EC ₅₀	half maximal concentration
ECMV	encephalomyocarditis virus
EcR	ecdysone receptor
Edg	endothelial differentiation gene
EGF	epithelial growth factor
ELC	EBV-induced molecule 1 ligand chemokine
ELISA	enzyme linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
Erk	extracellular signal related kinase
F (aa)	phenylalanine
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluorescein 5(6)-isothiocyanate
FMLP	formyl-methionine-leucine-phenylalanine
FTY720	2-amino-2-[2-(4-octylphenyl)ethyl]-propane-1,3-diol
g	gram
g	gravity
G (nt)	guanine
G protein	guanine nucleotide binding protein
G2A	G2 accumulation G protein-coupled receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanosine 5'-diphosphate
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	guanosine 5'-triphosphate
GTP γ S	guanosine 5'-[γ -thio]triphosphate
HA	haemagglutinin
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell
I (aa)	isoleucine
i.e.	<i>id est</i> (that is)
Ig	immunoglobulin
IPTG	isopropyl β -D-thiogalactopyranoside
IRES	internal ribosome entry site
IUPHAR	International Union of Pharmacology
IVS	intervening sequence

k	kilo
K (nt)	guanine or thymine
L	litre
LB medium	Luria-Bertani medium
LC-MS	liquid chromatography-mass spectrometry
LPA	lysophosphatidic acid
LPC	lysophosphatidylcholine
LPP1	lipid phosphate phosphohydrolase 1
LTB ₄	leukotriene B ₄
m	milli
M	molar
MAP	mitogen activated protein
MC	melanocortin
mGlu1 α	metabotropic glutamine 1 α receptor
MMLV	Moloney Murine Leukaemia Virus
mol	mole
MOPS	3-morpholinopropanesulphonic acid
M _r	molecular mass
mRNA	messenger RNA
MuA	muristerone A (2 β ,3 β ,5 β ,11 α ,14 α ,20R,22R-heptahydroxycholest-7-en-6-one)
n	nano
N (aa)	asparagine
N (nt)	adenine, cytosine, guanine or thymine
NCBI	National Centre for Biotechnology Information
NF- κ B	nuclear factor κ B
NGF	nerve growth factor
NK	natural killer
nt	nucleotide
OGR1	ovarian cancer G protein-coupled receptor 1
p	pico
psi	pounds per square inch
P ₂ Y ₁	P ₂ purinoceptor type Y ₁
PAF	platelet activating factor
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PC12	pheochromocytoma 12
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
pH	negative logarithm of the concentration of hydrogen ions
phS1P	phytosphingosine-1-phosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
plt	paucity of lymph node T cells
PonA	ponasterone A (2 β ,3 β ,14 α ,20R,22R-pentahydroxy-5 β -cholest-7-en-6-one)
PTx	pertussis toxin
PVDF	polyvinylidene difluoride
Q (aa)	glutamine

R (aa)	arginine
R (nt)	adenine or guanine
RAMP	receptor activity modifying protein
RGS	regulators of G protein signalling
RH	rat hepatoma
rpm	revolutions per minute
RPMI	Roswelli Park Memorial Institute
RSV	rous sarcoma virus
RXR	retinoid X receptor
S (aa)	serine
S (nt)	guanine or cytosine
S1P	sphingosine-1-phosphate
SDS	sodium dodecyl sulphate
SEM	standard error of mean
siRNA	short interfering RNA
SK	sphingosine kinase
SLC	secondary lymphoid tissue chemokine
SLC-1	somatostatin-like receptor 1
SPC	sphingosylphosphorylcholine
SPP	sphingosine-1-phosphate phosphatase
SST ₁	somatostatin type 1 receptor
SST ₂	somatostatin type 2 receptor
T	tera
T (aa)	threonine
T (nt)	thymine
TDAG8	T-cell death-associated gene 8
TM	transmembrane domain
TNF- α	tumour necrosis factor α
TRAF2	TNF receptor-associated factor 2
uv	ultraviolet
V2	vasopressin 2 receptor
vzg-1	ventricular zone gene 1
Y (aa)	tyrosine
Y (nt)	cytosine or thymine
μ	micro

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Chapter 1 – Introduction

1.1 General introduction

The activity of virtually every cell in the body is regulated by extracellular stimuli, which provide signals that are transmitted via a complex array of plasma membrane-associated receptors and intracellular proteins. A large proportion of these cell-surface receptors, including the S1P₄ receptor which is the topic of this thesis, belong to the G protein-coupled receptor (GPCR) superfamily. In this chapter, the GPCR family will be introduced before the S1P₄ receptor is considered within the context of the Edg receptor family, of which it is a member. The nature of the ligands which act at S1P₄ and their routes of production *in vivo* will be reviewed and the aims of this thesis will be outlined.

1.2 G protein-coupled receptors

1.2.1 Introduction

G protein-coupled receptors are integral membrane proteins which are found in a wide variety of organisms, including yeast [Marsh and Herskowitz, 1988] and the slime mould *Dictyostelium discoideum* [Ueda *et al.*, 2001], the plant *Arabidopsis thaliana* [Josefsson and Rask, 1997] and higher mammals such as man. The GPCR superfamily represents one of the largest protein families in the human genome. The first human GPCR was cloned in 1986 [Dixon *et al.*, 1986] and since this event, research in the field has continued and the range of techniques which can be employed in the analysis of the functions and properties of receptors has expanded. GPCRs are the subject of much industrial research since they are involved in many normal physiological and pathophysiological

processes and it has been estimated that 50 % of marketed drugs exert their actions via interactions with GPCRs [Howard *et al.*, 2001].

1.2.2 Structural features of GPCRs

GPCRs share common structural elements which may be illustrated by analysis of their amino acid sequences. Each GPCR sequence contains seven distinct regions of hydrophobic residues, which are believed to form α -helices and span the cellular membrane. These helices are connected by intracellular and extracellular loops comprised of hydrophilic amino acids. The amino-terminal region of a GPCR is located outside of the cell, whilst the carboxy-terminal tail resides intracellularly. Figure 1.1 shows a schematic representation of a GPCR located within the plasma membrane.

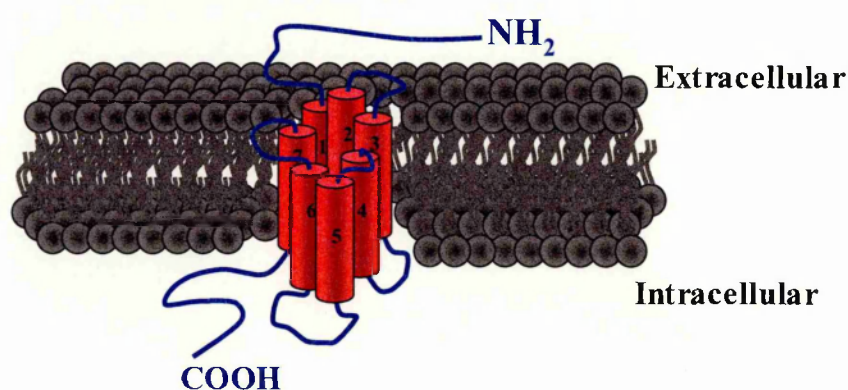


Figure 1. 1

Schematic representation of a GPCR residing in the plasma membrane.

This diagram illustrates the seven membrane-spanning α -helices of a GPCR. These are linked via intra- and extracellular loop regions. The amino-terminal tail of the receptor is located extracellularly whilst the carboxy-terminal tail is situated intracellularly.

The highest degree of sequence homology between GPCRs is observed within the transmembrane (TM) regions and each TM domain is comprised of between approximately 20-27 amino acids. The sequences of the loops and tails of

different GPCRs tend to be far more divergent. The size of the amino-terminal region varies and can contain between ten and 500 residues, whilst the loop sections can consist of as few as five amino acids to greater than 200 residues and the carboxy-terminal tail between 12 and 400 residues. In spite of their common structural elements, GPCRs are activated by myriad structurally diverse molecules including photons, odourants, neurotransmitters, nucleotides, lipids and amines, to polypeptides and proteins.

Direct study of the three-dimensional structures of GPCRs has not been as straightforward as it has been for cytosolic proteins such as non-receptor tyrosine kinase enzymes, due to the transmembrane spanning nature of GPCRs. Much of what was known about GPCR structure was inferred from studies of bacteriorhodopsin (reviewed by Khorana, 1988; Gether, 2000), a light activated proton pump expressed in the salt-loving archaebacterium *Halobacterium halobium*. This protein possesses seven α -helices, which span the membrane and, like vertebrate rhodopsin, uses the molecule retinal as a chromophore. In contrast, however, to mammalian GPCRs, bacteriorhodopsin is not coupled to G proteins and shares little sequence similarity with known GPCRs.

In 2000, the structure of rhodopsin, the first GPCR to be crystallised, was recorded at a resolution of 2.8 Å [Palczewski *et al.*, 2000]. The crystal structure of this inactive receptor was a highly organised heptahelical transmembrane bundle, with the seven α -helices arranged in an anticlockwise manner when viewed from the extracellular perspective. This structure has provided an improved template for the analysis of GPCR structure-function studies and computational modelling of receptors for use in drug discovery activities.

1.2.3 Classification of GPCRs

Over recent years, estimates of the number of GPCRs in the human genome have ranged from several hundred to greater than 1000. It is possible to categorise GPCRs into “endoGPCRs”, which are activated by endogenous molecules, and “chemosensory GPCRs”, which respond to exogenous stimuli such as odours and pheromones. A recent survey calculated there to be 367 endoGPCRs in the human genome, of which the majority have known ligands [Vassilatis *et al.*, 2003]. The remaining GPCRs have no known ligand and are therefore classified as orphan receptors. There has been much interest in the identification of ligands for these orphan receptors, since many may represent novel therapeutic targets (reviewed by Stadel *et al.*, 1997; Murphy *et al.*, 1998; Wilson *et al.*, 1998; Howard *et al.*, 2001).

GPCRs may be classified into four distinct categories using phylogenetic analysis. Family A (rhodopsin-like) contains the majority (284) of endoGPCRs, which are activated by peptides (e.g. chemokine, bradykinin and orexin receptors) and small molecules such as neurotransmitters (e.g. dopamine, serotonin and acetylcholine receptors) and lipids (e.g. S1P, LPA and cannabinoid receptors) or were classed as orphan receptors (98). Family B (secretin/glucagon) comprises 50 GPCRs, of which 16 are activated by peptides; the remainder were orphans. Family C (metabotropic glutamate) receptors number 17, including 6 orphans, whilst family D receptors numbered 10 with no orphans [Vassilatis *et al.*, 2003].

Of the four families, family A, to which the S1P receptor family belongs, is the

largest and best characterised. A phylogenetic tree of some family A receptors is shown in Figure 1.2.

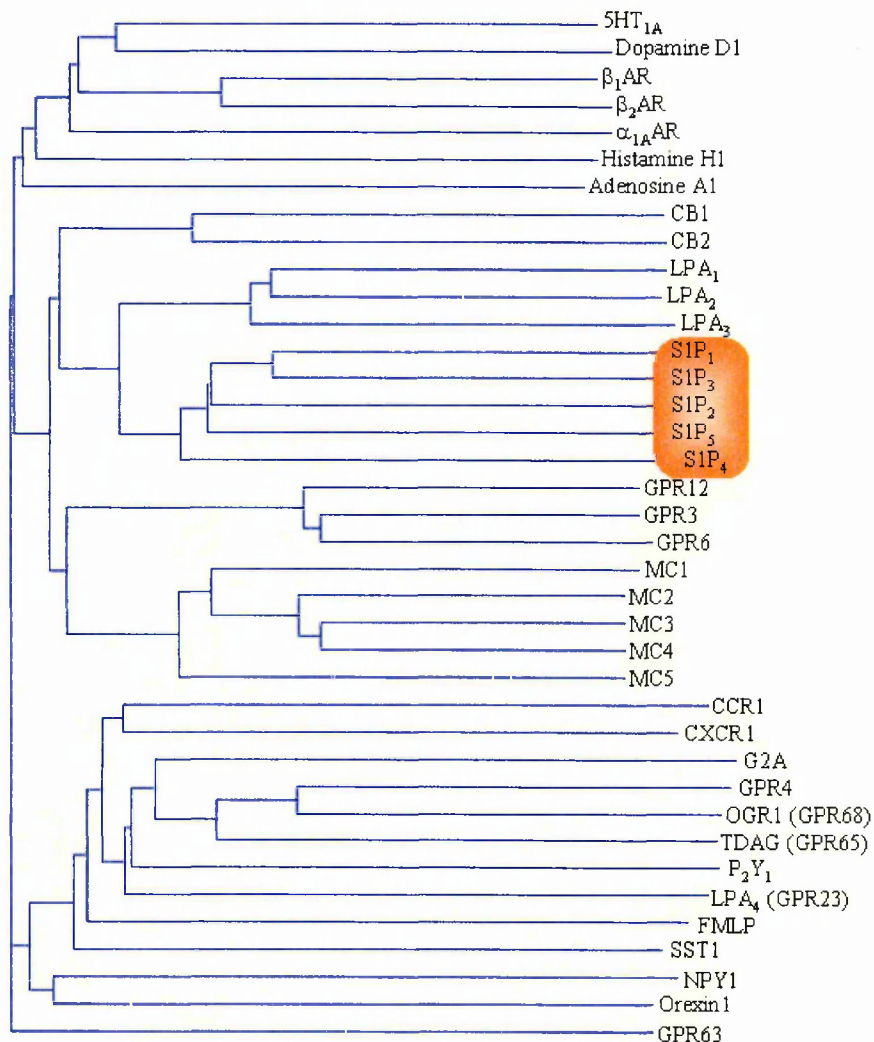


Figure 1. 2
Phylogenetic tree of selected family A GPCRs.

A number of highly conserved motifs are found within family A GPCRs, even though the overall homology between family members is quite low. This receptor family contains several proline residues present in TM domains IV, V, VI and VII, which probably introduce kinks into the helices. The conserved

D/ERY motif is located at the intracellular end of TM III and appears to be important in receptor activation. The arginine residue of this amino acid sequence is the only residue to be absolutely conserved throughout family A. Many family A receptors possess two cysteine residues: one present within the second extracellular loop and the other located at the top of TM III. The disulphide bridge which forms between these two residues serves to subdivide this extracellular loop into two smaller loops and may function to constrain the transmembrane bundle.

The extracellular amino-terminal tail of family A receptors varies in length and frequently contains a number of N-linked glycosylation motifs (N-X-T/S). The role of this post-translational modification remains unclear but may influence transportation of protein to the cell membrane after its synthesis in the endoplasmic reticulum as well as stabilising the protein on the cell-surface [Fiedler and Simons, 1995]. The length of the carboxy-terminal intracellular tail is also highly variable but it is typically rich in serine and threonine residues, which are sites for phosphorylation by intracellular kinases. The intracellular tail of family A GPCRs also contains one or more cysteine residues: these are sites of palmitoylation which causes tethering of the C-terminal tail to the cytoplasmic face of the plasma membrane to create a fourth intracellular loop [Wess, 1998].

The site of ligand binding for family A receptors is dependent on the nature of the ligand. Small ligands such as biogenic amines, purines, eicosanoids and lipids are believed to bind within the transmembrane crevice whilst peptide and protein ligands appear to interact with the extracellular loop and tail regions of the receptor.

1.2.4 Activation of GPCRs

1.2.4.1 Basic receptor theory

Ligands of GPCRs possess two fundamental properties: affinity and efficacy. The term affinity describes how well that ligand binds to the receptor, whilst the property of efficacy depicts the extent of the change in activation status of the receptor induced by that ligand. It is possible to determine the affinity of a ligand for a receptor using a technique such as radioligand binding, which allows calculation of the apparent dissociation constant (K_d) of that ligand for that receptor. If that ligand induces a functional response after binding, it is considered to display positive efficacy and is therefore defined as an agonist. In the current study, the term efficacy is used in the context of relative efficacy, and provides a description of the maximal response induced by that agonist, expressed as a percentage of that achieved by a full agonist (in this case S1P for the S1P₄ receptor). An agonist that exhibits an relative efficacy of less than 100 % is termed a partial agonist. Functional assays such as [³⁵S]GTPγS binding, adenylyl cyclase activation and receptor internalisation provide a means to measure the consequences of a ligand binding to a receptor and are used to determine the potency of a ligand. Agonist potency is described by the EC₅₀ value, which represents the concentration of agonist required to elicit 50 % of the maximal response in that system. If a ligand merely binds a receptor and in so doing, prevents activation of that receptor by an agonist, it has zero efficacy and is therefore classed as an antagonist. In recent years, many molecules that were thought to be antagonists have been reclassified as inverse agonists. This reflects the ability of a receptor system to display activity in the absence of agonist stimulation (the phenomenon of constitutive activity, discussed in this chapter,

section 2.4.3). An inverse agonist possesses negative efficacy and is able to reduce receptor constitutive activity (reviewed by Milligan *et al.*, 1995; de Ligt *et al.*, 2000).

1.2.4.2 Conformational changes associated with GPCR activation

Despite the diversity amongst ligands which activate members of the GPCR superfamily, the underlying fundamental mechanism of activation of these proteins appears to have been conserved throughout evolution. This allows a receptor activated by a small molecule to stimulate the same intracellular signalling pathways via the same class of G proteins, as a receptor activated by a protein. Regardless of the location of the ligand binding pocket, GPCR activation is achieved via conformational change.

GPCRs appear to preferentially exist in an inactive conformation, which is likely to be the result of evolutionary pressure and is mediated by constraining intramolecular interactions. This concept resulted from a landmark study of the α_{1b} -adrenergic receptor [Kjelsberg *et al.*, 1992] in which mutation of A²⁹³ to any other amino acid conferred a constitutively active phenotype to this receptor. It was subsequently shown that a constitutively active mutant of the β_2 -adrenergic receptor was structurally unstable. Hence it seemed probable that receptor activation as a consequence of agonist binding or the introduction of a constitutively activating mutation reflected the release of inactivating constraints.

The highly conserved D/ERY motif present within TM III of family A receptors may be involved in initiating the conformational change which occurs as a consequence of GPCR activation. Protonation of the aspartic or glutamic acid residue within this motif promotes rotation of TM VI [Gether, 2000]. Movement

of this transmembrane domain, along with TM III and possibly TM VII seems to be critical for the transition of a GPCR to the activated state (reviewed by Wess, 1998; Gether, 2000).

1.2.4.3 Constitutive activity of GPCRs

Traditional receptor theory proposed the existence of a single quiescent receptor state in which binding of an agonist promoted a conformational change in the receptor to yield the activated, or functional state. Recent evidence suggests that GPCRs have the potential to be active in the absence of agonist stimulation; this phenomenon is known as constitutive activity (reviewed by Leurs *et al.*, 1998; de Ligt *et al.*, 2000; Gether, 2000).

The discovery of agonists which possessed negative efficacy (defined in this chapter, section 2.4.1) implied the existence of a pre-existing equilibrium between two receptor states, in which one receptor state was associated with G proteins (and hence may be thought of as being active) and the second state was as a free (or inactive) receptor. Based on this assumption, the mode of action of the inverse agonist was to inhibit the spontaneous interaction of the receptor with G protein.

In a landmark study of receptor constitutive activity, replacement of residues 266-272 of the β_2 -AR with the homologous region of the α_{1b} -AR resulted in agonist-independent receptor signalling [Samama *et al.*, 1993]. The authors determined that the established ternary complex model of receptor activation was unable to mathematically describe the experimental results obtained with the constitutively active β_2 -AR. The ternary complex model was therefore extended so that it could account for constitutive activity. This model (shown in Figure

1.3) assumes that the receptor exists in an equilibrium between two states, the uncoupled inactive receptor (R) and the active state (R*). Only the active R* form is capable of binding G protein and so the state AR*G represents the sole ternary complex which may be formed. The extent of spontaneous isomerisation between R and R* is described by the isomerisation constant (J). An increase in the term J therefore accounts for enhanced basal activity observed with constitutively active receptors.

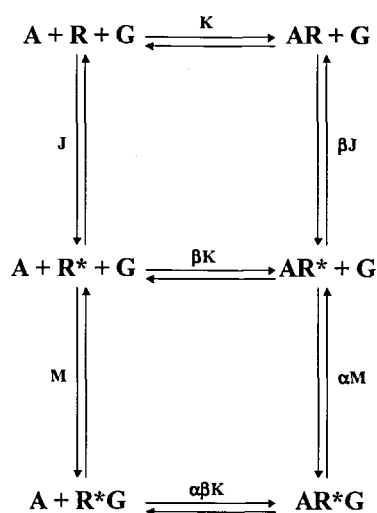


Figure 1. 3

Schematic representation of the extended ternary complex model.

This theoretical model describes the interactions of receptor which is considered to exist in equilibrium between the resting state (R) and the active state (R*), with agonist (A) and G protein (G). Equilibria are described by the various constants shown.

Reproduced from [Samama *et al.*, 1993].

Contrary to the traditional concepts, where receptors were thought to be “on” or “off”, it seems likely that GPCRs are mobile proteins which are subject to constant local unfolding and refolding reactions. Each of these reactions has the capacity to subtly change the receptor conformation and hence a GPCR may be considered to exist in an ensemble of conformational states [Kenakin, 2002].

This range of conformations may be represented by a Gaussian distribution. In the absence of agonist, this population may be considered to exist in the “inactive” state. After agonist stimulation, the population shifts and is in the “G protein bound state” which allows activation of G protein and hence propagation of the signal. These two states may intersect (Figure 1.4) and the population of receptors within this intersection may spontaneously activate G protein without the need for agonist activation and hence represent the property of GPCR constitutive activity [Kenakin, 2002].

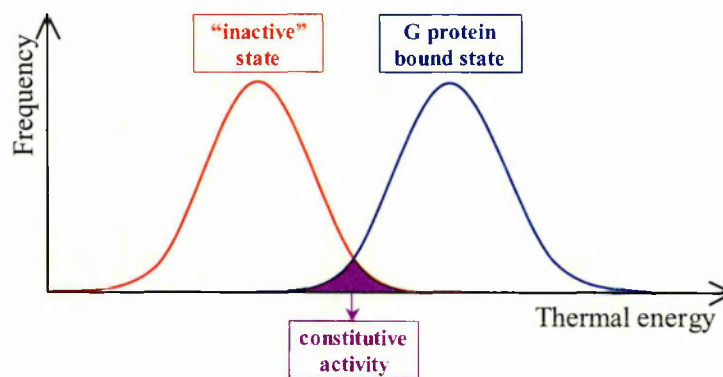


Figure 1. 4
GPCRs exist in an ensemble of microstates.

The inactive (non-signalling) and G protein bound (signalling) states are shown as two normal distributions. The shaded intersection between the two ensembles represents the conformations common to the two states. Receptors nominally in the inactive state that fall within this intersection can spontaneously activate G protein and hence represent GPCR constitutive activity.

Even though only a small proportion of the population of states of a given receptor may be in the constitutively active conformation, it is often possible to detect agonist-independent signalling simply by over-expressing that receptor. It has been suggested that many GPCRs possess a degree of basal activity and that receptor density is directly related to constitutive activity. Many studies of this nature have been reported (for example see Casarosa *et al.*, [2001]). The

physiological relevance of constitutively active receptors is unclear, but it may be related to the concept of tonic regulation of physiological systems by receptors. Tonic regulation had previously been explained by the presence of an endogenous agonist and its continued interaction with that receptor system; however, the realisation that receptors possess constitutive activity sheds new light on this idea. It is tempting to speculate that physiological tone mediated by GPCRs may be mediated by receptor basal activity as an addition to, or instead of, the action of endogenous agonists.

1.2.5 Heterotrimeric G proteins

Guanine nucleotide binding proteins (G proteins) couple membrane bound GPCRs to their cellular effector systems. GPCR-mediated signal transduction is considered to progress via activation of G proteins. Recently however, it was reported that a mutant of the angiotensin II type 1 (AT1a) receptor which lacked the ability to couple to G proteins was able to activate the ERK pathway [Seta *et al.*, 2002]; so it is possible that a GPCR can still transmit information to cells even if interaction with their cognate G protein is prevented.

Heterotrimeric G proteins have been said to be “involved in everything from sex in yeast to cognition in humans” [Marx, 1994]. The G protein heterotrimer comprises α , β and γ subunits, which are the products of distinct genes. When stimulated, G proteins undergo a cycle of activation and deactivation in which the α subunit and $\beta\gamma$ heterodimer are able to interact with their reaction partners (such as the GPCR itself, guanine nucleotides and effector molecules). During this cycle, the inactive, GDP bound G protein heterotrimer interacts with an activated receptor. This causes dissociation of bound GDP from the $G\alpha$ subunit,

which is replaced by GTP present within the cellular cytosol. Binding of GTP promotes a conformational change within the $G\alpha$ subunit, causing dissociation of this subunit from the $\beta\gamma$ heterodimer and allowing interaction of the GTP-bound $G\alpha$ and $\beta\gamma$ subunits with effector molecules. Intrinsic GTPase activity of the $G\alpha$ subunit cleaves the terminal phosphate group from the bound GTP, converting it to GDP and permitting reassociation of the $G\alpha$ G protein with the $\beta\gamma$ heterodimer, which causes deactivation of the G protein subunits.

At least 20 G protein α subunits, encoded by 17 different genes, have been identified. These may be divided into the four families: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{12/13}$, which couple to different effector systems (summarised in Table 1.1).

Family	Effector systems regulated
$G\alpha_s$	Stimulates adenylyl cyclase isoforms
$G\alpha_{i/o/t}$	Further divided into $G\alpha_{i/o/z}$ and $G\alpha_{t/g}$ sub groups $G\alpha_{i/o/z}$ subunits inhibit some adenylyl cyclase isoforms $G\alpha_{t/g}$ subunits: $G\alpha_t$ stimulates retinal phosphodiesterase; $G\alpha_g$ presumably stimulates a gustatory effector
$G\alpha_q$	Activates β -isoenzymes of phospholipase C and non-receptor Btk family tyrosine kinase enzymes
$G\alpha_{12/13}$	Regulates low molecular weight Rho family G proteins

Table 1. 1
Summary of G protein α subunit actions.

Activation of adenylyl cyclase and formation of cAMP is the most well established paradigm for second messenger generation in response to GPCR activation and is mediated by the $G\alpha_s$ class of G protein. Activation of a $G\alpha_{i/o}$ subunit inhibits cAMP formation via inhibition of adenylyl cyclase. There are three distinct $G\alpha_i$ species: $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$. Subunits of the $G\alpha_{q/11}$ class couple to various isoforms of phospholipase C (PLC) and include $G\alpha_q$, $G\alpha_{11}$,

$G\alpha_{14}$ and $G\alpha_{15/16}$. The $G\alpha_{12/13}$ family appear to be involved in activation of the low molecular weight G protein, Rho (reviewed by Sah *et al.*, [2000]).

Activation of $G\alpha$ and $G\beta\gamma$ subunits can affect the activity of a whole host of effector molecules and/or ion channels. Thus, the pattern of effects which result from receptor activation are complex. Additionally, cells may express multiple receptors that respond to the same ligand by activating opposing signalling pathways. It has also been suggested that the actions of different agonists at the same receptor can activate different G proteins (the phenomenon of agonist trafficking, [Kenakin, 2002; Hermans, 2003]).

Clearly, the ability of a given GPCR to propagate a signalling response via activation of a particular G protein requires interaction between receptor and G protein. Investigations into the regions of the receptor involved in mediating such interactions have been obtained through the analysis of hybrid receptors constructed between functionally distinct GPCRs. Amino acids present within the second and third intracellular loops appear to be essential to confer selectivity of G protein recognition [Wess, 1998; Hamm, 2001]. Similarly, efforts have been made to understand the areas of the $G\alpha$ subunit which form contacts with the active GPCR. The five carboxy-terminal amino acids of $G\alpha$ subunits are key in controlling the specificity of receptor-G protein coupling [Wess, 1998]. Chimeric $G\alpha$ G proteins in which these five amino acids are replaced with those from a different class of G protein have been used to stimulate pathways that would not normally be activated by that particular receptor [Milligan and Rees, 1999; Kowal *et al.*, 2002]. For example, expression of a $G\alpha_{q/i}$ chimera (which is a $G\alpha_q$ backbone containing the final five amino acids from $G\alpha_i$) permits

stimulation of PLC β by the dopamine D2 or adenosine A1 receptors, which normally couple exclusively to G α_i G proteins [Wess, 1998].

Many G α subunits have been shown to be expressed almost ubiquitously in mammalian cells, with exceptions such as G α_o , which is expressed in brain, and G α_{16} , expression of which is restricted to B and T cells [Offermanns and Simon, 1995; Grant *et al.*, 1997]. The murine G protein, G α_{15} and its human orthologue, G α_{16} , are promiscuous G proteins which are thought to be able to couple to most GPCRs [Stables *et al.*, 1997] and hence are often employed when attempts are made to assign ligands to orphan receptors. The likelihood of the orphan receptor coupling to the promiscuous G protein is high and any activation of the receptor may be conveniently measured using calcium flux assays.

Various exogenous agents can interfere with the interaction between G proteins and GPCRs, or can modulate the activity of the G protein itself. Such reagents may be useful in the elucidation of the nature of G proteins activated by a particular receptor. The bacterium *Bordetella pertussis*, the causative agent of whooping cough, produces a number of toxins of which one, pertussis toxin (also named islet activating protein), inactivates G proteins of the G α_i class via ADP-ribosylation. This covalent modification serves to prevent interaction of an agonist- (or constitutively-) activated receptor with GDP-bound G protein. However, pertussis toxin treatment does not affect the intrinsic ability of the G α subunit to exchange guanine nucleotides or to hydrolyse GTP (reviewed by Milligan [1988]). The location of G α_i G protein subunit ADP-ribosylation by pertussis toxin is a conserved cysteine residue present near the carboxy-terminus

of the protein. Mutation of this residue to any other amino acid has been shown to confer resistance to the actions of this toxin [Bahia *et al.*, 1998].

Another exotoxin, in this case isolated from culture supernatants of *Vibrio cholerae*, modulates $G\alpha_s$ G protein activity. Like pertussis toxin, cholera toxin also possess ADP-ribosyltransferase activity which catalyses the transfer of ADP-ribose to an arginine residue present in the $G\alpha_s$ subunit. This modification stabilises the GTP-bound form of the α subunit of G_s , and as the intrinsic GTPase activity of the subunit is inhibited, the $G\alpha_s$ subunit is in a permanently activated state.

1.2.6 Termination of signalling at GPCRs

Activation of a GPCR leads to its interaction with G protein, which becomes stimulated as a result of this interaction. In its active conformation, a GPCR may stimulate many G protein molecules resulting in amplification of the initial stimulus. Negative regulation of responses to agonists is clearly necessary to allow control of such systems.

The lifetime of the signalling event is dictated by the longevity of the $G\alpha$ -GTP species, which is dependent on the intrinsic GTPase activity of the $G\alpha$ subunit. This activity promotes hydrolysis of GTP back to GDP and allows reassociation of the $G\alpha$ subunit with the $\beta\gamma$ heterodimer to form the inactive, GDP-bound heterotrimeric G protein. However, this is not normally sufficient to account for the rapid termination of signalling which is observed in most systems. Recently, the actions of a family of RGS (regulators of G protein signalling) proteins have been shown to further attenuate signalling mediated via $G\alpha_i$ or $G\alpha_q$ G proteins.

Interaction between RGS proteins and the GTP-bound $G\alpha$ subunit causes a significant increase (100-1000 fold) in the GTPase activity of the target $G\alpha$ subunit and therefore silences the G protein-signalling pathway (reviewed by Hepler, 1999; Zhong and Neubig, 2001).

Signalling is also commonly regulated at the level of the GPCR itself via the phenomenon of receptor desensitisation. There are two types of desensitisation: homologous and heterologous, each of which will be briefly considered.

Homologous desensitisation describes the specific attenuation of signalling arising from an activated receptor. The active receptor conformation is a target for phosphorylation by members of the G protein-coupled receptor kinase (GRK) family (reviewed by Buenemann and Hosey, 1999; Penn *et al.*, 2000; Ferguson, 2001). These kinases phosphorylate serine and threonine residues present in the third intracellular loop and carboxy-terminal tail of the active receptor, which permits binding of β -arrestin proteins [Ferguson, 2001]. β -arrestin appears to function as an adapter protein to link the receptor with components of the clathrin internalisation machinery. Subsequent to GPCR phosphorylation and binding of β -arrestin, receptors may be sequestered from the plasma membrane. Once internalised, the GPCR may be resensitised, dephosphorylated and recycled back to the plasma membrane or targeted to lysosomes for degradation. The fate of the internalised GPCR appears to depend on the arrangement of serine residues in the carboxy-terminal tail of the receptor. Receptors which possess a serine cluster within this region are internalised and recycle slowly (e.g. vasopressin V2 receptor) whereas receptors which lack such a cluster are rapidly dephosphorylated and recycled back to the cell-surface (e.g. β_2 AR)

[Oakley *et al.*, 1999]. In addition to their primary role in GPCR densitisation, there is some evidence that β -arrestins can also act as scaffold proteins and may facilitate GPCR-mediated activation of the mitogen activated protein (MAP) kinase pathway [McDonald *et al.*, 2000]. It has also been suggested that agonist-dependent phosphorylation of GPCRs on serine/threonine residues may be mediated by casein kinase 1 α in addition to the actions of GRKs (reviewed by Tobin [2002]).

The process of heterologous desensitisation is independent of stimulation of that receptor by an agonist and is achieved via receptor phosphorylation by the serine/threonine kinases, protein kinase A (PKA) and protein kinase C (PKC), which leads to loss of receptor function.

1.3 The Edg family of GPCRs

1.3.1 Introduction to Edg receptors

In 1990, an abundant transcript induced in phorbol ester-treated differentiating endothelial cells was cloned and found to encode a 381 amino acid orphan GPCR [Hla and Maciag, 1990]. The gene product was named Endothelial differentiation gene 1 (Edg1) and was the first member of this new receptor subfamily. Even before the ligand for this receptor had been identified, it was suggested that Edg1 may be involved in angiogenesis since it was an immediate-early gene induced in cultured human umbilical vein endothelial cells (HUVEC). An elegant study used the yeast two-hybrid system to demonstrate the ability of the third intracellular loop of Edg1 to associate with G α_i G proteins hence suggesting the pathway that may be stimulated by this orphan receptor. [Lee *et al.*, 1996]. The subsequent observation that Edg1-transfected HEK-293 cells

formed a network of cell-cell aggregates when grown in FCS-containing medium, whilst untransfected cells maintained normal morphology under the same conditions led to the identification of sphingosine-1-phosphate (S1P) as the putative endogenous agonist for this receptor [Lee *et al.*, 1998]. The related Edg2 receptor, which had been cloned from a neuronal cell cDNA library, had already been identified as a receptor for lysophosphatidic acid (LPA) [Hecht *et al.*, 1996]. These findings suggested that the remaining orphans within this receptor family would be activated by these ligands and that the Edg receptors represented a new receptor family, which were activated by the lysophospholipids S1P and LPA. Further Edg family members were cloned and studies of these GPCRs conducted by many groups have confirmed their identity as high affinity receptors for S1P or LPA (reviewed by Fukushima *et al.*, 2001; Kluk and Hla, 2002; Takuwa *et al.*, 2002). A total of eight family members have been identified and phylogenetic analysis of GPCRs from the human genome predicted that there are no further Edg receptors [Vassilatis *et al.*, 2003].

The ligands for the Edg receptor family are structurally similar. S1P and LPA (shown in Figure 1.5) both contain a polar head group with a long chain fatty acid tail. The Edg receptors are most closely related to the cannabinoid (CB) receptor family (shown in the phylogenetic tree in Figure 1.2), and share approximately 25 % sequence identity with CB receptors. The endogenous ligands of the cannabinoid receptors, anandamide and 2-arachidonoylglycerol, have structures which are similar to S1P and LPA since they also comprise a polar head group and long aliphatic tail. In common with the CB receptors, Edg family GPCRs lack the commonly conserved cysteine residue present within the first extracellular loop of most family A receptors (previously discussed in this

chapter, section 2.3). These common features might suggest that Edg and CB receptors evolved from a common ancestral gene.

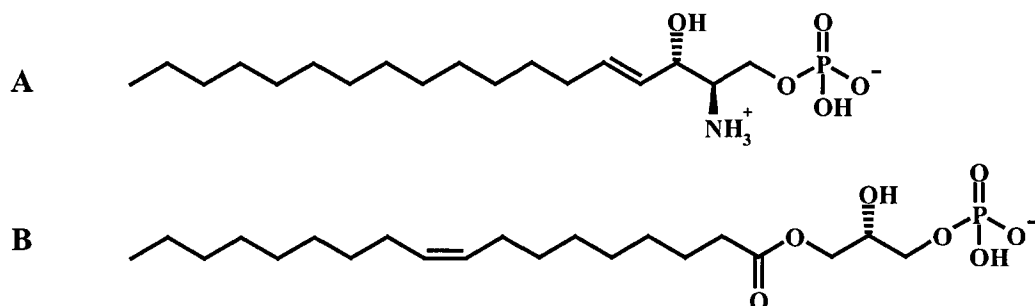


Figure 1. 5
Structures of Edg family ligands.

- A Sphingosine-1-phosphate (S1P)
B Lysophosphatidic acid (LPA)
(shown here is 18:1 LPA)

The signalling characteristics of the Edg receptor family have been extensively studied and the G proteins to which each receptor couples has been determined. Each Edg receptor couples to $\text{G}\alpha_i$ G proteins and with the exception of Edg1, they couple to additional G protein families (summarised in Table 1.2).

Receptor	Ligand	G protein coupling
Edg1	S1P	G_i
Edg2	LPA	$\text{G}_i, \text{G}_q, \text{G}_{12/13}$
Edg3	S1P	$\text{G}_i, \text{G}_q, \text{G}_{12/13}$
Edg4	LPA	$\text{G}_i, \text{G}_q, \text{G}_{12/13}$
Edg5	S1P	$\text{G}_i, \text{G}_q, \text{G}_{12/13}$
Edg6	S1P	$\text{G}_i, \text{G}_{12/13}$
Edg7	LPA	G_i, G_q
Edg8	S1P	$\text{G}_i, \text{G}_{12/13}$

Table 1. 2
Summary of G protein activation by Edg family receptors.

1.3.2 Classification of Edg receptors

The colloquial name “Edg”, which has been used to describe this family of receptors was originally used to describe the immediate early response gene product cloned from HUVEC, Edg1 [Hla and Maciag, 1990]. Other orphan receptors were cloned and given various trivial names (e.g. vzg-1 for Edg2) and it became clear that the term Edg bore no relevance to the other seven receptors. The Edg family members were renamed in 2002, in accordance with IUPHAR guidelines, whereby a receptor is named using an abbreviation for the natural agonist with the highest potency, followed by a subscript Arabic numeral [Chun *et al.*, 2002]. The order of the numbering of the reclassified Edg receptors was also chosen to reflect the chronology of publication of the novel receptor sequences. Table 1.3 shows the new names of the Edg receptors.

IUPHAR name	Edg name	Previous names	Ligand
S1P ₁	Edg1		
S1P ₂	Edg5	H218, AGR16	
S1P ₃	Edg3		S1P
S1P ₄	Edg6		
S1P ₅	Edg8	nrg-1	
LPA ₁	Edg2	vzg-1, rec1.3	
LPA ₂	Edg4		LPA
LPA ₃	Edg7		

Table 1.3
Summary of Edg family receptor classification consistent with IUPHAR guidelines.

Phylogenetic analysis of the Edg receptor family reveals that the receptors form two clusters, which correspond to the ligands by which they are activated (Figure 1.6).

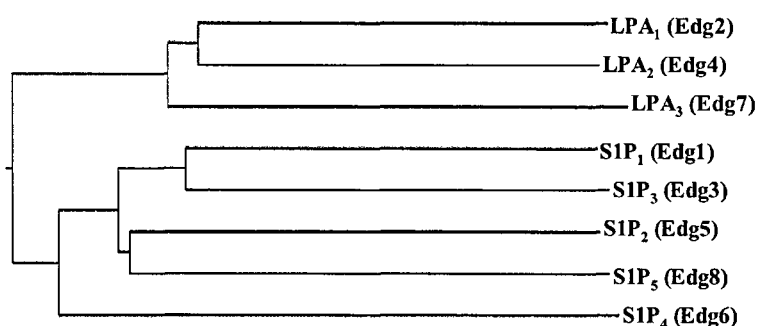


Figure 1. 6

Phylogenetic tree of Edg family receptors.

Comparison of sequences of Edg family GPCRs reveal the receptors form two clusters, which correspond with ligand preference. The Edg family was renamed to reflect ligand preference exhibited by the receptors (in accordance with IUPHAR guidelines) and the new nomenclature is used here.

1.3.3 The S1P receptor family

The S1P receptor family has been the subject of several comprehensive reviews (for example see, Pyne and Pyne, 2000a; Takuwa *et al.*, 2001; Takuwa *et al.*, 2002; Kluk and Hla, 2002; Siehler and Manning, 2002). S1P receptors are found in many organisms, including the puffer fish *Takifugu rubripes* [Yamaguchi *et al.*, 1999] and the sea squirt *Ciona intestinalis*, which indicates the highly conserved nature of GPCR-mediated S1P signalling. Characterisation of the members of the S1P receptor has revealed that these highly specific S1P receptors mediate different responses to S1P and that cells may express multiple S1P receptors which activate opposing pathways (summarised in Figure 1.7). Dissection of the pharmacology of the individual S1P receptors has been hampered by the expression of endogenous S1P receptors on most cell types and the lack of receptor selective agonists or antagonists. Progress is being made in this area however, and a recent publication described the identification of an S1P_{1/3} selective agonist [Im *et al.*, 2001a].

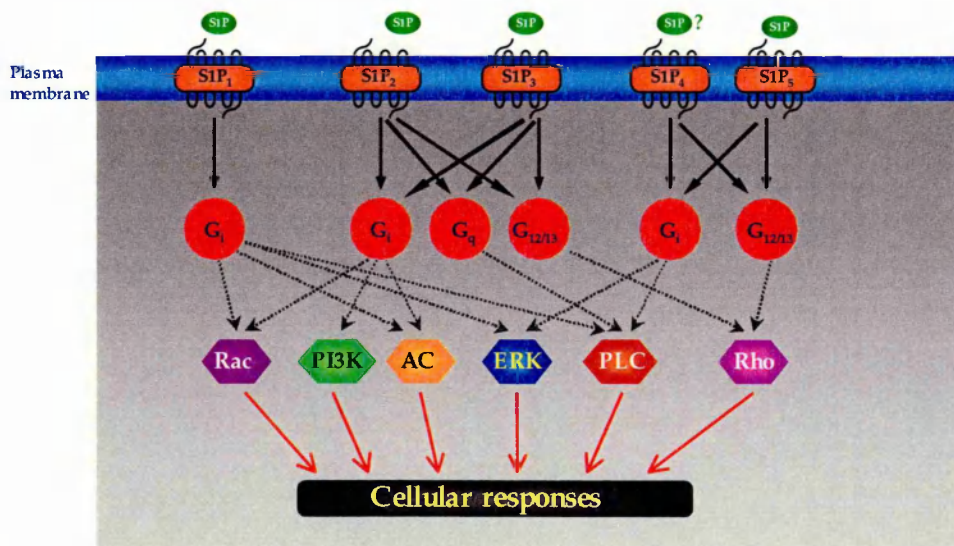


Figure 1. 7

Signal transduction by S1P family receptors.

S1P, present in serum binds to members of the S1P receptor family to activate the various G proteins shown. Different S1P receptors couple to different G protein subfamilies (e.g. S1P₁ activates G_i whilst S1P₂ is linked to G_i, G_q and G_{12/13}). G protein activation leads to stimulation or inhibition of various downstream intracellular pathways, some of which are shown here.

Reproduced and updated from Spiegel and Milstien [2003].

Since it is the prototypical S1P receptor, S1P₁ has been the most extensively studied member of this receptor family. The S1P₁ receptor, which was previously named Edg1 and has been introduced in this chapter, section 3.1, is a 381 amino acid protein, the mRNA of which is widely expressed. The human S1P₁ gene is located on chromosome 1p21. As described previously in this chapter, overexpression studies defined S1P₁ as a high affinity S1P receptor ($K_d = 8.1 \text{ nM}$) [Lee *et al.*, 1998]. The related molecule, dihydrosphingosine-1-phosphate, which lacks the double bond present within the S1P backbone, was also able to bind and activate this receptor. High concentrations of the related molecule sphingosylphosphorylcholine (SPC) were able to displace S1P, in common with its effects as a low affinity ligand for other S1P receptors. Rapid internalisation of the receptor occurs after stimulation with S1P which is

followed by recycling of the receptor back to the plasma membrane [Liu *et al.*, 1999]. Additionally, Kohno *et al.*, [2002] showed that internalisation of this receptor was dependent on receptor glycosylation since mutant S1P₁ which could not be glycosylated was poorly internalised in response to S1P. Agonist-dependent phosphorylation of S1P₁ has been found to be mediated by GRK-2 and the extreme carboxy-terminus of the receptor was shown to be the target for the actions of this kinase. Phosphorylation of residues within this region was required for receptor internalisation [Watterson *et al.*, 2002].

Many different cellular responses to S1P have been shown to depend on S1P₁. S1P₁ couples to G α_i G proteins and stimulation of the receptor with S1P promotes activation of ERK, inhibition of adenylyl cyclase and Rac-dependent chemotaxis. Reports describing the ability of S1P₁ to stimulate mobilisation of calcium are conflicting and may suggest that this response is cell type dependent [Siehler and Manning, 2002]. Activation of S1P₁ positively regulates proliferative/survival and migratory signalling pathways in many cell types. Endothelial cells migrate towards S1P in a manner which requires phosphorylation of S1P₁ by Akt [Lee *et al.*, 2001], although it is unlikely that other S1P receptors would be Akt substrates as the residue of S1P₁ which is phosphorylated is not conserved throughout this receptor family. Binding of S1P to S1P₁ expressed on endothelial cells has been shown to stimulate endothelial nitric oxide synthase (eNOS), resulting in production of the potent vasodilator, nitric oxide (NO) [Igarashi and Michel, 2001].

Disruption of the S1P₁ gene in mice caused embryonic lethality between E12.5 and E14.5. This was due to the lack of vascular smooth muscle cell recruitment

to the developing vasculature and demonstrated a clear role for S1P₁ in vascular maturation [Liu *et al.*, 2000]. Specific deletion of the S1P₁ gene in endothelial cells of mice using the conditional Cre/LoxP system produced a highly similar phenotype to that of the S1P₁ knockout. It was suggested the phenotype of the S1P₁^{-/-} mice was due to the loss of function of this receptor in endothelial cells, rather than vascular smooth muscle cells, since deletion of S1P₁ in vascular smooth muscle cells did not affect vascular maturation [Allende *et al.*, 2003].

Interestingly, the phenotype of the S1P₁^{-/-} mice was similar to that caused by deletion of platelet-derived growth factor (PDGF- β) or its receptor (PDGFR- β) in mice. This suggested that vascular maturation may involve cross-talk between S1P₁ and the PDGF- β receptor. This concept was supported by the observations of Hobson *et al.*, [2001] who showed that PDGF-stimulated cell motility in HEK-293 cells was dependent on S1P₁ expression. The authors proposed a sequential model in which PDGF activates sphingosine kinase promoting release of S1P, which acts as an autocrine mediator to stimulate S1P₁ thereby activating the small G protein Rac which is required for cell motility. Studies by Alderton *et al.*, [2001] reported that PDGFR- β forms a tethered complex with S1P₁ in HEK-293 cells and is involved in cell proliferation. Within this complex, efficient stimulation of the ERK pathway by S1P and PDGF was reported to occur through a mechanism that involves PDGR- β tyrosine phosphorylation of G α_i , which is released upon S1P₁ stimulation.

S1P₂ (Edg5) was independently cloned from rat by two groups and named AGR16 or H218 [Fukushima *et al.*, 2001]. The human gene, located on chromosome 19p13.2, encodes a 352 amino acid protein and S1P was shown to

be a high affinity agonist ($K_d = 27$ nM) of this receptor [Van Brocklyn *et al.*, 1999]. The S1P₂ transcript is abundantly expressed in heart and lung, is detected at lower levels in brain and is expressed more strongly in embryonic brain. Transfection of cells with S1P₂ induces cell rounding and neurite retraction in response to S1P [Van Brocklyn *et al.*, 1999]. S1P₂ couples to multiple ($G\alpha_i$, $G\alpha_q$, $G\alpha_{12/13}$) G proteins and can also activate Rho and induce stress fibre formation. In contrast to S1P₁, which stimulates Rac and promotes cell movement, S1P₂ is chemorepellant, inhibiting Rac and blocking migration to S1P [Takuwa, 2002].

Targeted deletion of the S1P₂ gene was reported to lead to neuronal dysfunction, although surprisingly there were no apparent anatomical or physiological abnormalities [MacLennan *et al.*, 2001]. Interestingly, the zebrafish gene *Mil* (Miles Apart), which closely resembles the mammalian S1P₂ gene, appears to be involved in cardiac development [Kupperman *et al.*, 2000].

The S1P₃ receptor (formerly called Edg3) was initially isolated from human DNA and mapped to chromosome 9q22.1-22.2 [Yamaguchi *et al.*, 1996]. The gene encodes a 378 amino acid protein which was shown to bind S1P with high affinity ($K_d = 26$ nM) [Kon *et al.*, 1999]. S1P₃ mRNA is expressed in many tissues including heart, brain, liver, lung, kidney, pancreas, thymus and spleen [Kluk and Hla, 2002]. S1P₃ couples to multiple G proteins to exert its effects and the ability of this receptor to stimulate $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$, but not $G\alpha_s$, has been demonstrated using [³⁵S]GTP γ S binding [Windh *et al.*, 1999]. Activation of S1P₃ by S1P promotes mobilisation of intracellular calcium via PLC activation, stimulation of ERK, cell migration, cytoskeletal organisation and

morphogenetic differentiation [reviewed by Kluk and Hla, 2002; Siehler and Manning, 2002]. In common with S1P₂, S1P₃ has been shown to activate nuclear factor κ B (NF- κ B) in response to S1P stimulation and this appears to reflect activation of G_q G proteins [Siehler *et al.*, 2001].

Deletion of the S1P₃ gene in mice did not produce any obvious phenotypic abnormality [Ishii *et al.*, 2001]. This suggested a non essential role for S1P₃ in murine development, however activation of PLC by S1P was significantly decreased in embryonic fibroblasts derived from S1P₃ ^{-/-} mice. Generation of mice null for both S1P₂ and S1P₃ caused reduced litter sizes and significant infant mortality; although those pups which survived lacked any obvious phenotype [Ishii *et al.*, 2002]. *In vitro* analysis of S1P signalling in S1P₂S1P₃ ^{-/-} embryonic fibroblasts showed severe defects in activation of Rho, PLC and calcium mobilisation. S1P receptor signalling therefore appears to be involved in perinatal survival and the signalling of S1P₂ and S1P₃ receptors is non-redundant.

The S1P₄ gene was cloned from *in vitro* differentiated dendritic cells and was originally named Edg6 [Graeler *et al.*, 1998]. This receptor will be discussed in more detail in this chapter, section 3.5.

The final S1P receptor, S1P₅ (previously named Edg8/nrg-1) was cloned from rat PC12 cells and encodes a 400 amino acid protein. S1P₅ was shown to be another high affinity S1P receptor ($K_d = 2$ nM [Im *et al.*, 2000a]) and has differential expression patterns in rat and human. The human S1P₅ gene is located on chromosome 19p13.2 and its mRNA has been detected in skeletal muscle, heart, and kidney with lower expression in liver and placenta; in contrast, rat S1P₅

mRNA was predominantly expressed in brain [Niedernberg *et al.*, 2002]. Activation of S1P₅ causes cell rounding, mobilisation of calcium, cell survival and inhibition of both adenylyl cyclase and ERK activation [Im *et al.*, 2000a; Niedernberg *et al.*, 2003a]. Differential effects on cell proliferation were observed between human and rat S1P₅: the rat receptor is antiproliferative whilst the human receptor is not [Niedernberg *et al.*, 2002]. S1P₅ can couple to G_i and G_{12/13} G proteins and was reported by Niedernberg *et al.*, [2003a] to be constitutively active. Interestingly, constitutive inhibition of adenylyl cyclase was enhanced by S1P-induced receptor activation but the ability of S1P₅ to inhibit basal ERK activity was not affected by S1P. These data were interpreted as suggesting that different signal transduction pathways are not equally activated through a constitutively active GPCR that couples to multiple G proteins. It is not clear whether this scenario is applicable to other S1P receptors.

The effect of deleting the S1P₅ gene in mice has not been reported, but given the reported differences in expression patterns between human and rodent forms of S1P₅ [Niedernberg *et al.*, 2002], it is unclear how useful analysis of S1P₅ null mice would be in understanding the function of the human receptor.

Significant progress has been made in elucidating the *in vivo* function of the various S1P receptors and the generation of mice null for S1P₁ demonstrated the critical involvement of the receptor in vascular maturation [Liu *et al.*, 2000]. Conditional knockout experiments and production of double knockout mice have been used to further address the question of the role of these receptors in physiology and disease. Additional work is required to fully unravel the significance of S1P receptor activation and to understand precisely how the

different receptor isoforms, which can have similar or opposing functions, control the diverse effects of S1P *in vivo*.

1.3.4 The LPA receptor family

The LPA receptor family comprises three closely related GPCRs, which bind LPA with nM affinity. A fourth GPCR has recently been designated as LPA₄ but this receptor is only distantly related and shares approximately 20 % sequence identity with the other LPA receptors (shown in Figure 1.2). This receptor (previously named P₂Y₉/GPR23) was found to bind LPA with high affinity (45 nM K_d) and couples to mobilisation of intracellular calcium and stimulation of adenylyl cyclase activity [Noguchi *et al.*, 2003]. This LPA receptor has evolved from a different ancestor than that of the LPA₁₋₃ receptors and the role of LPA₄ *in vivo* is currently unclear.

Unlike the S1P receptors, the LPA₁₋₃ receptors contain an intron located within the middle of TM VI, the location of which is absolutely conserved between human and mouse [Contos and Chun, 1998; Contos and Chun, 2000; Contos and Chun, 2001]. This suggests that these LPA receptors were derived from a common ancestral gene. Significant progress has been made in this area since the identification of the first LPA receptor. Most notably, synthetic LPA mimetics have been developed, some of which show receptor subtype-selective activities [Im *et al.*, 2000b], whilst others act as competitive antagonists [Sardar *et al.*, 2002].

The ligand, LPA can be produced by several distinct enzymatic mechanisms. Intracellularly, the sequential actions of phospholipase D (which converts phosphatidylcholine to phosphatidic acid) and phospholipase A1 and A2

enzymes (which deacylate phosphatidic acid) yield LPA. Phospholipase A2 may also be secreted from cells to produce extracellular LPA. An alternative route of production of extracellular LPA is via the lysophospholipase D enzyme, autotaxin, which acts within plasma to hydrolyse lysophosphatidylcholine and is believed to represent the principal route of production of extracellular LPA. Bioactive LPA is dephosphorylated by enzymes of the lipid phosphate phosphohydrolase family, some of which may possess ecto-activity. Finally, LPA may be produced from PA (and *vice versa*) by the action of the LPA acyl transferase enzyme.

LPA is released from activated platelets and is present in serum at low μM concentrations. Although the term LPA is used to refer to the single entity 1-oleoyl LPA, it actually describes a family of molecules which can contain different fatty acyl chains with different levels of saturation. LPA is a potent signalling molecule which is involved in many physiological and pathophysiological process such as cell differentiation and proliferation, induction of apoptosis, cytoskeletal arrangements and cell invasion [reviewed by Ye *et al.*, 2002]. LPA affects a wide variety of cell types and reflects the widespread expression of the various LPA receptors. The LPA receptor family has been recently reviewed [Fukushima *et al.*, 2001; Fukushima and Chun, 2001]; each receptor will now be briefly discussed.

LPA₁ was identified as a result of studies designed to identify novel GPCRs associated with neuron production in mouse cerebral cortex. It was named ventricular zone gene (vzg-1) and assigned as the second Edg family member since it was similar to the orphan Edg1 (S1P₁) receptor. The human LPA₁ gene

is located on chromosome 9p31.3-32 and encodes a protein of 364 amino acids [An *et al.*, 1997; Fukushima and Chun, 2001]. Analysis of heterologously expressed LPA₁ revealed that stimulation of the receptor caused cell proliferation, Rho-mediated actin rearrangement and stimulation of serum response elements via activation of G α_i , G α_q and G $\alpha_{12/13}$ G proteins. The receptor was found to be widely expressed in the murine embryonic nervous system where it may play a role in neurogenesis and is also expressed in adult central nervous system (CNS) where it may be involved in the biology of myelinating cells. Receptor mRNA is also expressed outside the CNS, predominantly in testis and intestine, and moderately in heart, lung, kidney, spleen, thymus, muscle and stomach, as well as in several cancers. Disruption of the LPA₁ gene in mice was expected to cause nervous system defects. Targeted deletion of this gene resulted in approximately 50 % neonatal lethality due to decreased suckling, possibly caused by olfaction defects [Contos *et al.*, 2000]. In addition, LPA₁^{-/-} mice were smaller and exhibited craniofacial abnormalities and increased apoptosis in peripheral nerves. This suggests a role for LPA₁ in nervous system development and survival of myelinating cells.

Functional LPA₁ has recently been detected at the cell nucleus [Gobeil *et al.*, 2003]. Stimulation of nuclear LPA₁ caused activation of G_{i/o} G proteins, mobilisation of nuclear calcium and induction of pro-inflammatory gene expression. This observation suggests that the nucleus may be a potential organelle for intracrine signalling by lysophospholipids. It will be interesting to discover whether other LPA or S1P receptors are also active at the cell nucleus.

The LPA₂ receptor, which is located on human chromosome 19p12, is a 351 amino acid GPCR that shares approximately 46 % sequence identity with LPA₁. Its sequence was discovered from database searches of human genomic DNA during attempts to identify LPA₁ receptor homologues [An *et al.*, 1998]. Several variants of this receptor have been detected in human cancer cells and in fact the original report describing the LPA₂ (Edg4) receptor had cloned and characterised a mutant from an ovarian cancer cell line [An *et al.*, 1998]. This clone contained a base deletion which caused a frame shift and resulted in a mutant that contained an extended carboxy-terminal tail [Contos and Chun, 2000]. Subsequent comparison with the wild type receptor did not reveal any differences in G protein coupling; both wild type and mutant forms of the receptor couple to G α_i , G α_q and G $\alpha_{12/13}$ G proteins [An *et al.*, 1998; Ishii *et al.*, 2000] and the role of mutant LPA₂ receptors in cancer is unclear. Activation of LPA₂ results in activation of PLC, stimulation of ERK, cell rounding and inhibition of adenylyl cyclase [Ishii *et al.*, 2000]. The gene is expressed in peripheral leukocytes, thymus, spleen, prostate and pancreas [An *et al.*, 1998]. Expression of LPA₁ and LPA₂ has been shown to change after T cell activation and these receptors transduce opposing effects on T cell motility which may be relevant *in vivo* [Zheng *et al.*, 2001]. LPA₂ knockout mice exhibited no obvious phenotypic abnormalities and failed to reveal any obvious behavioural, anatomical or histological defects [Contos *et al.*, 2002a]. Indeed, the same report describes analysis of mice null for both LPA₁ and LPA₂, which displayed essentially the same phenotype as LPA₁ $\text{--}/\text{--}$ mice. This suggests that the LPA₁ and LPA₂ receptors have redundant functions in mediating multiple LPA responses.

The discovery of a third LPA receptor was independently reported by two groups [Bandoh *et al.*, 1999; Im *et al.*, 2000b]. The LPA₃ gene is present on human chromosome 1p22.3-31.1 [Contos and Chun, 2001] and the resultant 353 amino acid receptor shares approximately 50 % sequence identity with LPA₁ and LPA₂. LPA₃ mRNA was found to be expressed in heart, pancreas, kidney, brain, prostate and testis, and less abundantly in ovary and lung [Bandoh *et al.*, 1999; Im *et al.*, 2000b]. An LPA₃ variant (HOFNH30) which is highly expressed in placenta has also been identified and may be a splice variant of LPA₃ [Fitzgerald *et al.*, 2000]. LPA-stimulation of LPA₃ promoted mobilisation of calcium from intracellular stores which was mediated via activation of G α_q G proteins and was dependent on activation of PLC [Im *et al.*, 2000b]. The receptor has also been reported to couple to G α_i G proteins although other groups have contradicted these findings [Fukushima *et al.*, 2001]. In marked contrast to the effects of LPA₁ and LPA₂ stimulation, activation of LPA₃ was found to promote neurite elongation and inhibited LPA-induced cell rounding in LPA_{1/2} expressing cells [Ishii *et al.*, 2000]. Analysis of *in vivo* LPA₃ function by deletion in mouse has not yet been reported and the functional role of this receptor remains unclear.

1.3.5 S1P₄ (Edg6)

S1P₄ (formerly named Edg6) was cloned in 1998 from *in vitro* differentiated dendritic cells, in an effort to discover novel chemokine receptors [Graeler *et al.*, 1998]. It was categorised as the sixth member of the then named Edg receptor family, on the basis of sequence similarity with known members of this family. The sequence of this gene coded a 384 amino acid protein, which bore the hallmarks of being a GPCR since it possessed seven putative transmembrane domains. The translated S1P₄ sequence also contained a putative N-

glycosylation site in the amino-terminal tail as well as several sites for potential modification by PKC phosphorylation and palmitoylation within the carboxy-terminal region [Graeler *et al.*, 1998]. The S1P₄ receptor shares greatest sequence identity with other S1P receptors and is less closely related to the LPA receptors, summarised in Table 1.4. It is also closely related to the cannabinoid receptors, sharing 20 % and 34 % sequence identity with CB1 and CB2, respectively.

Receptor	Sequence identity with S1P ₄
S1P ₁	39 %
S1P ₂	36 %
S1P ₃	40 %
S1P ₅	37 %
LPA ₁	30 %
LPA ₂	35 %
LPA ₃	30 %

Table 1. 4
Sequence identity between S1P₄ and other S1P and LPA receptors.

S1P₄ has a very distinct pattern of mRNA expression and is principally detected within the immune system. This is in marked contrast to that of the other S1P receptors (detailed in this chapter, section 3.3) and suggested that S1P₄ may play a role in immune function. More recent studies of S1P₄ expression have added to the initial findings, and are summarised in Table 1.5. In addition, the study of [Vassilatis *et al.*, 2003] detected low levels of S1P₄ mRNA in murine cerebral tissues (e.g. brain stem, hippocampus, striatum, thalamus). Since no other studies have detected this transcript in these tissues, the significance of these findings is unclear.

The pattern of S1P₄ expression in various human tissues has been investigated using a polyclonal anti-S1P₄ antibody in an immunohistochemistry study. The findings are summarised in Table 1.5 and Figure 1.8 shows some examples of S1P₄-expressing cells present within tissue sections.

Whilst significant advances were made with the other S1P receptors, progress with S1P₄ was slow. Based on its similarity with other S1P receptors S1P was a candidate S1P₄ ligand, but attempts to demonstrate this experimentally were frustrated by reported difficulties in expressing functional receptor [Im *et al.*, 2001a]. However, in 2000, two independent groups showed that S1P bound to S1P₄ with an apparent K_d of 12-63 nM [Van Brocklyn *et al.*, 2000; Yamazaki *et al.*, 2000]. Van Brocklyn *et al.*, [2000] expressed S1P₄ in HEK293 cells and detected specific binding of S1P to the recombinant receptor. Bound S1P could be displaced by competition with the related lipids dihydroS1P (dhS1P) and sphingosylphosphorylcholine (SPC), although SPC could only compete for S1P binding when used at high (μ M) concentrations. This was in broad agreement with the ligand preference exhibited by other S1P receptors, except for the relatively low affinity with which S1P₄ bound S1P. S1P₄ expressed in CHO-K1 cells demonstrated pertussis toxin-sensitive activation of ERK in response to S1P, indicating that S1P₄ coupled to G α_i G proteins. The study of Yamzaki *et al.*, [2000] also detected specific binding of S1P which could be displaced by high concentrations of SPC. Additionally, this group reported that stimulation of S1P₄ with S1P induced mobilisation of intracellular calcium via activation of the phospholipase C (PLC) enzyme. This response was partially sensitive to pertussis toxin treatment which suggested coupling of the receptor to G α_i and

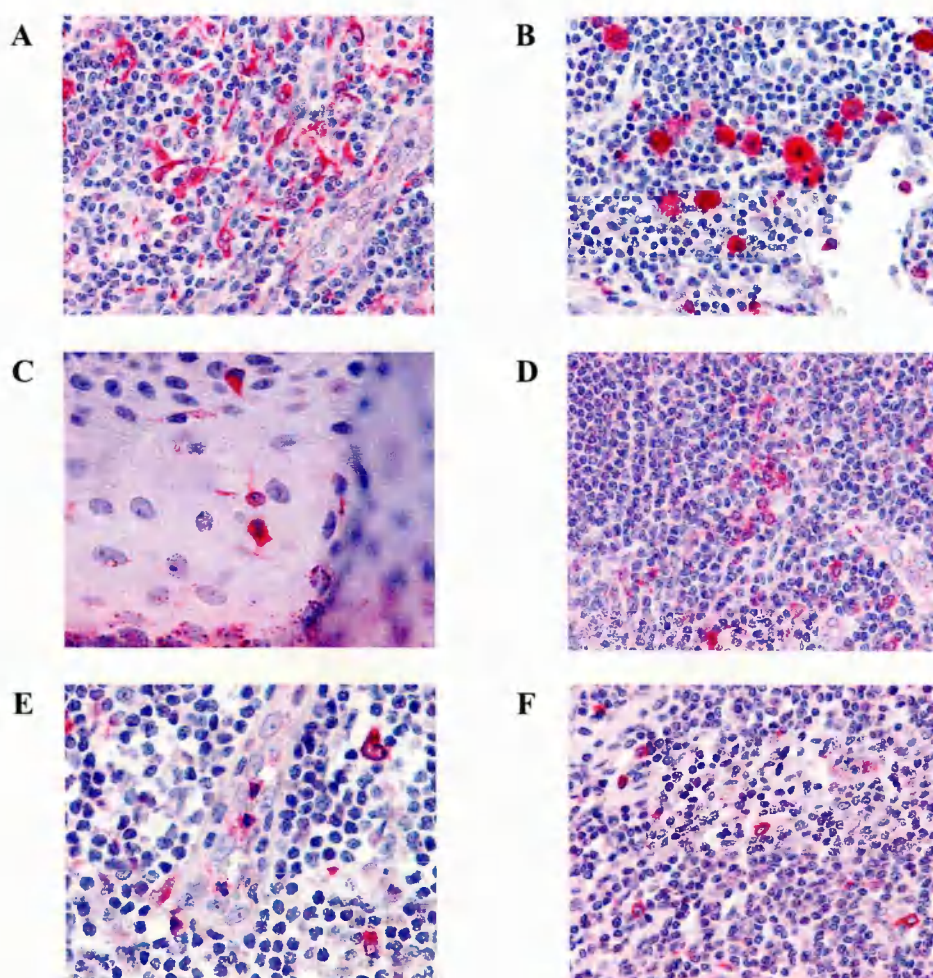
Tissue/Cell Type	Relative level	Method	Species	Reference
spleen	+++	Northern blot	human	1
thymus	++			
peripheral leukocyte	+++			
lymph node	++			
bone marrow	++			
foetal spleen	+++			
foetal liver	+			
foetal thymus	+			
foetal lung	+			
naïve CD4 T cell	N.A.	RT-PCR, Western blotting	mouse	2
kidney	+	RT-PCR	mouse	3
lung	+++			
ovary	+			
peripheral leukocyte	+++			
prostate	+			
skin	++			
spleen	++			
stomach	++			
thymus	++			
thyroid	+			
lung	+++	Northern blot	mouse	4
spleen	+++			
thymus	+			
peripheral monocytes	++	RT-PCR	human	5
<i>in vitro</i> differentiated macrophages	++			
platelet	++	RT-PCR	human	6
polymorphonuclear leukocytes	+++			
lymphocytes (subsets of)	+ to +++	immunohisto- chemistry	human	7
mast cells	+++			
granulocytes	+++			
Langerhans cells	+++			
lymph node interdigitating cells	+++			
neutrophils	++			
eosinophils	++			

Table 1.5**Summary of tissues and cells reported to express S1P₄.**

Note: “relative level” refers to the level of signal obtained within that experiment and it would not be appropriate to compare relative S1P₄ expression levels taken from different studies/publications.

+ faint signal; ++ medium signal; +++ strong signal; N.A. not appropriate.

- References:
- ¹ Graeler *et al.*, 1998
 - ² Graeler and Goetzl, 2002
 - ³ Vassilatis *et al.*, 2003
 - ⁴ Contos *et al.*, 2002b
 - ⁵ Fueller *et al.*, 2003
 - ⁶ Motohashi *et al.*, 2000
 - ⁷ In house studies

**Figure 1. 8****Expression of S1P₄ detected in tissue sections.**

Immunohistochemical studies have been performed using a rabbit polyclonal antibody, which recognises the human S1P₄ protein. Affinity purified antiserum was used at a dilution of 1:250 on paraffin-embedded, formalin-fixed tissues. Cells positive for S1P₄ expression stained fuschia-red. Tissue sections were also stained with haematoxylin and eosin. The identity of S1P₄ positive cells was defined by histological analysis. This study was performed by LifeSpan Biosciences, Inc. (Seattle, WA., U.S.A.).

- A Normal lymph node showing strong staining of interdigitating cells (40x)
- B Inflamed lymph node showing strong staining of mast cells (40x)
- C Inflamed skin showing strong staining of Langerhans cells (60x)
- D Normal lymph node showing staining of lymphocytes within interfollicular zone (40x)
- E Inflamed lymph node showing staining of neutrophils (60x)
- F Small intestine with Peyer's patches showing staining of lymphocytes (40x)

possibly $G\alpha_q$ G proteins. However, untransfected cells were also observed to mobilise calcium in response to S1P stimulation, although the magnitude of response was smaller than for the S1P₄-transfected cells. The ability of this receptor to promote a calcium flux has not been confirmed by other laboratories and therefore remains unclear.

Analysis of the S1P₄ gene in mouse, found it to be present as a single copy comprising two exons with an intronless coding region, located on central chromosome 10, which is syntenic with human chromosome 19. The presence of a single, uninterrupted coding region is similar to that observed for the other S1P receptor genes and supports the theory that an ancestral S1P gene diverged to yield S1P₄ and the other S1P receptor genes [Contos *et al.*, 2002b]. Within the same study, it was reported that the S1P₄ gene is arranged in tandem with the murine $G\alpha_{16}$ orthologue, $G\alpha_{15}$ on mouse chromosome 10. Expression of the $G\alpha_{15}$ and S1P₄ genes primarily in haematopoietic tissues and their chromosomal proximity suggests that these transcripts may be co-expressed in the same cells. The authors therefore suggested that co-expression of $G\alpha_{15/16}$ and S1P₄ proteins might imply this G protein to be the native coupling partner of S1P₄ and that stimulation of S1P₄ would result in increased concentration of intracellular calcium [Contos *et al.*, 2002b]. Direct analysis of the G proteins activated by S1P₄ was performed by Graeler *et al.*, [2003] and revealed that this receptor coupled to $G\alpha_i$ and $G\alpha_{12/13}$ subunits, but not $G\alpha_q$ or $G\alpha_{15/16}$. These observations were interpreted as suggesting that the previously reported tandem genomic arrangement of the S1P₄ and $G\alpha_{15/16}$ genes may not be functionally relevant. In agreement with the well documented ability of this receptor to couple to $G\alpha_i$ G

proteins, the same study observed pertussis toxin-sensitive activation of PLC by S1P₄. Activation of S1P₄ was also shown to potently activate Rho and promote stress fibre formation. The authors suggested this reflected the ability of stimulated S1P₄ to cause pronounced activation of G $\alpha_{12/13}$ which could be relevant to the *in vivo* functions of this receptor [Graeler *et al.*, 2003]. S1P₄ has also recently been shown to promote cell migration via activation of Cdc42 [Kohno *et al.*, 2003].

There is some uncertainty as to whether S1P represents the true endogenous ligand for S1P₄. This lipid binds to the S1P₄ receptor with significantly lower affinity than that with which it binds other S1P receptors and is a relatively poor agonist of S1P₄. In addition, phylogenetic analysis of the S1P receptor family (Figure 1.6) reveals that although this receptor family forms a discrete cluster, S1P₄ is on the edge of this cluster, suggesting that it may be activated by a structurally distinct lipid. The molecule, phytosphingosine-1-phosphate (phS1P) was recently identified as a high affinity ligand for the S1P₄ receptor, and is discussed further in this chapter, section 4.3.

There is still little data regarding the physiological role of the S1P₄ receptor within the immune system. This is partly a consequence of the lack of receptor selective agonists, combined with the expression of multiple S1P receptor subtypes on haematopoietic and lymphoid cells. Disruption of the S1P₄ gene in mice may indicate the processes in which the function of this receptor is required.

1.3.6 Other lipid GPCRs

LPA and S1P receptors are not the only GPCRs to respond to lipid agonists. In addition to the well characterised GPCRs for platelet activating factor (PAF), cannabinoids and prostaglandins, several orphan receptors have recently been reported to respond to lipids which are structurally similar to S1P and LPA.

The ovarian cancer GPCR 1 (OGR1) receptor (previously named GPR68) is closely related to the PAF receptor and P₂Y purinergic receptors but shares little homology with Edg family GPCRs. The gene was cloned from an ovarian cancer cell line and is expressed in placenta, lung, liver, testis, spleen, small intestine and peripheral leukocytes [Xu *et al.*, 2000]. OGR1 is activated by nM concentrations of the lipid SPC, which had previously been identified as a low affinity S1P receptor agonist. Binding of SPC to OGR1 stimulated activation of G_i and G_q G proteins, resulting in mobilisation of intracellular calcium, activation of ERK and inhibition of cell proliferation [Xu *et al.*, 2000]. The related GPR65 receptor, named T cell death-associated gene 8 (TDAG8) is expressed in spleen, lymph node and peripheral leukocytes [Im *et al.*, 2001b]. This receptor shares 41 % identity with OGR1 and has been shown to respond to μ M concentrations of the glycosphingolipid, psychosine. Stimulation of TDAG8 with psychosine promoted pertussis toxin-insensitive inhibition of cAMP, which was suggested to reflect activation of G α_z G proteins [Im *et al.*, 2001b]. The transcriptionally regulated gene, G2A (G2 accumulation G protein-coupled receptor), is predominantly expressed in lymphocytes and is also closely related to OGR1 and TDAG8. Genetic studies of this then orphan receptor suggested a role in peripheral lymphocyte homeostasis, since mice null for G2A developed secondary lymphoid organ enlargement and late-onset autoimmune syndrome

[Le *et al.*, 2001]. Subsequently, the serum lipids lysophosphatidylcholine (LPC) and SPC were identified as G2A receptor agonists (K_d values of 65 nM and 230 nM, respectively). The phenotype of the G2A knockout was particularly interesting given that the G2A receptor ligand, LPC is implicated in the pathogenesis of the autoimmune disease systemic lupus erythematosus. Stimulation of the receptor with LPC promoted mobilisation of intracellular calcium, activation of ERK and cell migration via $G\alpha_i$ G proteins [Kabarowski *et al.*, 2001]. Another GPCR closely related to the OGR1, TDAG8 and G2A receptors, GPR4, also responds to SPC and LPC. In contrast to the profile of ligand preference exhibited by G2A, GPR4 binds SPC with higher affinity (36 nM) than it does LPC (159 nM) [Zhu *et al.*, 2001]. This receptor was found to couple to $G\alpha_i$ G proteins and stimulate activation of ERK, mobilisation of calcium from intracellular stores and DNA synthesis. These four lipid receptors form a distinct cluster (shown in Figure 1.2) and may represent a new lysophospholipid subfamily.

S1P has recently been shown to activate several non Edg family GPCRs. The closely related GPR3, 6 and 12 orphan receptors are predominantly expressed in cerebral tissues and are most similar to Edg and cannabinoid receptors (shown in Figure 1.2), sharing approximately 42 % sequence identity with these receptors. GPR3, 6 and 12 are constitutively active receptors and stimulate opposing $G\alpha_s$ and $G\alpha_i$ G proteins. Treatment with low concentrations of S1P was found to further stimulate these receptors and although their physiological role is not currently understood, they have been identified as three additional S1P receptors [Uhlenbrock *et al.*, 2002].

The mammalian orthologue of the *Xenopus laevis* PSP24 receptor, GPR63 was recently reported to be a low affinity S1P receptor [Niedernberg *et al.*, 2003b]. This receptor is expressed in stomach, small intestine and central nervous system and does not share significant sequence homology with Edg or other S1P receptors (Figure 1.2).

The existence of multiple receptors which respond to lysophospholipids and elicit diverse effects reinforces the importance of lipid mediators in biology.

1.4 Sphingolipid signalling

Sphingolipids are a major component of cell membranes and the sphingolipid metabolites ceramide, sphingosine and S1P play important roles in the regulation of cell proliferation, survival and death. The organisms in which these processes occur are diverse and include mammals, yeast and plants.

1.4.1 Sphingosine and ceramide

The bioactive lipids sphingosine and ceramide generally inhibit cell proliferation and promote apoptosis. The molecule sphingosine was named in 1884 after the Sphinx to reflect “the many enigmas which it has presented to the enquirer” [Smith and Merrill, 2002]. Ceramide may be produced via *de novo* synthesis or from the precursor sphingomyelin whilst sphingosine is produced via deacylation of ceramide by the family of ceramidase enzymes. The levels of ceramide and sphingosine found in resting cells are generally low since their production is tightly regulated.

1.4.2 Sphingosine-1-phosphate

S1P (D-*erythro*-sphingosine-1-phosphate) is the principal phosphorylated sphingoid base in mammalian cells. In contrast to the pro-apoptotic actions of its precursors, ceramide and sphingosine, S1P is a cell survival factor. This bioactive lipid regulates a range of cellular responses, including cytoskeletal changes, cell movement, cell proliferation, angiogenesis and vascular maturation. S1P was originally thought to be an intracellular second messenger but the discovery of the S1P family of GPCRs, which bound this lipid with high affinity, redefined S1P as an extracellular receptor agonist. Despite the clear role for cell-surface S1P receptors in mediating many effects of S1P, the suggestion that this lysophospholipid also functions as an intracellular signalling molecule remains. Whilst some of the evidence to support this is compelling, the absence of identified intracellular targets has led to considerable debate in this area. The actions of S1P have been the subject of numerous reviews (for example see Pyne and Pyne, 2000a; Pyne and Pyne, 2000b; Maceyka *et al.*, 2002; Spiegel *et al.*, 2002; Pyne and Pyne 2002; Hla, 2003; Spiegel and Milstien, 2003). Many of the micro-effects of S1P have already been discussed in the context of the GPCRs which respond to S1P (this chapter, section 3). The current section will discuss the mechanisms controlling S1P production and breakdown as well as the macro-effects induced by this bioactive lipid and the concept of S1P as a dual intracellular and extracellular signalling molecule.

As is common with many signalling molecules, S1P levels in cells tend to be low and are balanced by an equilibrium between its synthesis and degradation (Figure 1.9). S1P is generated via the phosphorylation of sphingosine, which is catalysed by the sphingosine kinase (SK) enzyme. There are two different routes

for S1P degradation: reversible dephosphorylation by S1P phosphatase, or irreversible degradation into hexadecenal and phosphoethanolamine via the actions of S1P lyase. Platelets are an exception and store high concentrations of S1P, which is not subject to degradation by S1P lyase since platelets do not express this enzyme. This stored S1P is released on cell activation and aggregation and represents the major source of S1P in serum and plasma.

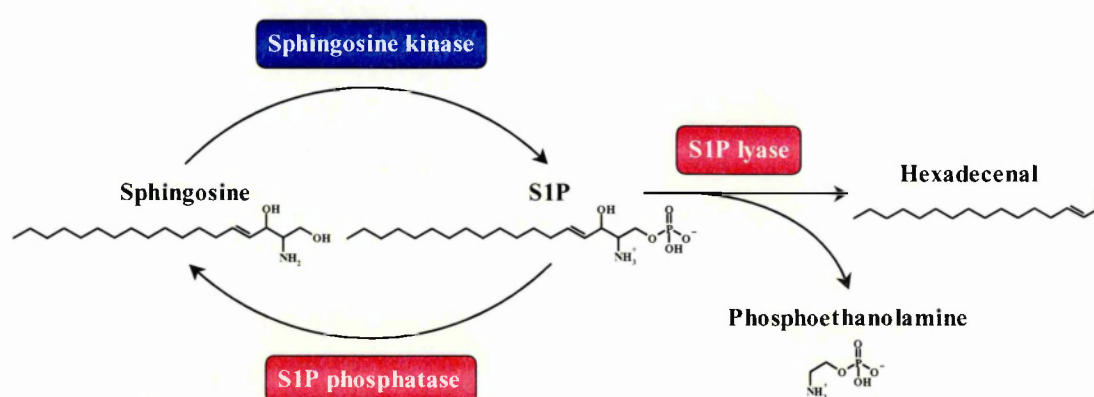


Figure 1. 9

Synthesis and degradation of S1P.

Sphingosine is converted to S1P by the actions of sphingosine kinase. Conversely, two different enzymes may degrade S1P: S1P phosphatase removes the phosphate to yield sphingosine; S1P lyase cleaves the C2-C3 bond of S1P to produce hexadecenal and phosphoethanolamine.

Extracellular S1P is albumin bound which is believed to prevent it from binding non-specifically to plasma membrane surfaces and is metabolically stable in this form [Yatomi *et al.*, 1997]. The concentrations of S1P in normal human plasma and serum have been reported to be approximately 200 nM and 485 nM, respectively [Yatomi *et al.*, 1997]. Release of S1P from activated platelets will significantly increase the concentration of circulating S1P. These concentrations are significantly higher than the dissociation constants for S1P binding to each isolated S1P-family receptor.

Most cells produce S1P intracellularly which reflects the widespread expression of sphingosine kinase. It is not clear exactly how S1P is transported out of cells and the presence of the polar headgroup suggests S1P will not readily traverse the plasma membrane; candidate transporters include the P-glycoprotein lipid transporter Abcb1 [Honig *et al.*, 2003]. S1P released from cells acts as an autocrine or paracrine mediator, stimulating cell-surface receptors on the cell from which it was released (autocrine) or on nearby cells (paracrine). In its putative intracellular role, S1P is proposed to function as a second messenger for growth factors and alters the activity of specific intracellular target proteins (discussed by [Pyne and Pyne, 2000b; Spiegel and Milstein, 2003]).

Sphingosine kinase catalyses formation of S1P and is a highly conserved enzyme present in organisms as diverse as plants, yeast, worms and mammals. It is activated in response to external stimuli such as growth and survival factors. Two human genes encoding sphingosine kinase enzymes have been cloned (SK1 and SK2), which are the products of distinct genes. SK1 and SK2 are cytosolic proteins of 384 and 618 amino acids, respectively, which contain five conserved catalytic domains and are most closely related to the diacylglycerol kinase family.

The SK1 enzyme was originally isolated from rat kidney [Olivera *et al.*, 1998]. The importance of S1P as a signalling molecule is underscored by the existence of SK orthologues in many organisms ranging from mammals to yeast, plants and flies. The sequence of SK1 contains putative calcium/calmodulin binding domains and consensus PKA and PKC phosphorylation sites, which may be involved in regulation of enzyme activity [Pyne and Pyne, 2000b]. Precise

studies of the role of these enzymes in cellular processes have been hampered by the lack of specific inhibitors. Whilst compounds such as dimethylsphingosine (DMS) competitively inhibit SK1, DMS also inhibits PKC, which complicates interpretation of results since PKC may modify the activity of SK1.

Although this enzyme is believed to be cytosolic and contains no signal sequence or TM domains, constitutive export of SK1 from endothelial cells has been observed [Ancellin *et al.*, 2002]. Many molecules have been reported to increase SK1 activity, including ligands for GPCRs, agonists of growth factor receptor tyrosine kinases such as PDGF, epithelial growth factor (EGF) and nerve growth factor (NGF), as well as tumour necrosis factor- α (TNF- α), phorbol esters and even S1P itself [Maceyka *et al.*, 2002; Pyne and Pyne, 2002]. The activity of SK1 is reported to be bimodal: prior to activation by exogenous stimuli the enzyme exhibits relatively high basal turnover which may be involved in housekeeping functions; after activation the catalytic activity of the enzyme increases approximately 2-fold resulting in a concomitant increase in S1P concentration [Pitson *et al.*, 2000]. In the same study, expression of a catalytically inactive dominant negative SK1 mutant prevented activation of SK1 in response to exogenous stimuli such as TNF- α and phorbol esters but did not affect basal SK1 activity in these cells. Activation of SK1 in response to TNF- α has been shown to involve a direct interaction between the TNF receptor-associated factor 2 (TRAF2) and SK1 [Xia *et al.*, 2002]. SK1 protects against apoptosis induced by ceramide, TNF- α , Fas ligand and other toxic agents via production of S1P, which inhibits the cytochrome-c release-dependent mitochondrial death pathway induced by these stimuli [Hla, 2003]. Production of S1P as a consequence of SK activation is accompanied by a decrease in

ceramide levels and the fate of a cell appears to depend on the relative levels of these molecules, rather than their absolute concentrations. This system is a conserved stress mechanism that has been referred to as the “sphingolipid rheostat”, shown in Figure 1.10.

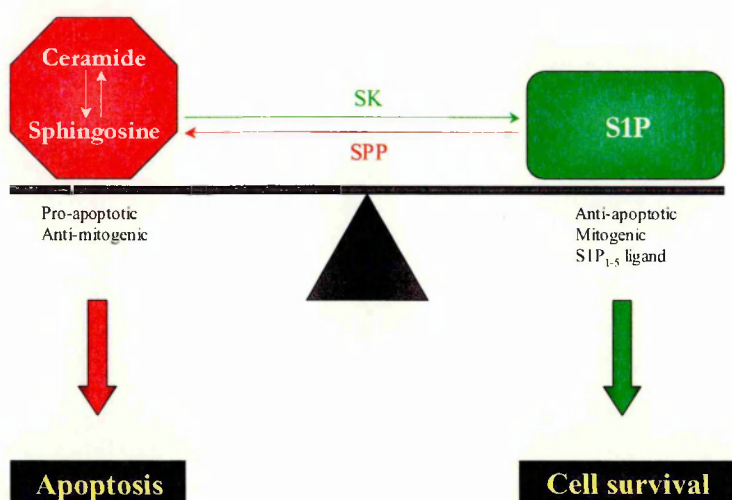


Figure 1. 10

The ceramide-S1P rheostat.

The sphingolipids ceramide, sphingosine and S1P are interconvertible and the balance between them determines cell fate. Ceramide and sphingosine are associated with arrest of cell growth and apoptosis whilst S1P promotes cell growth and survival.

The related SK2 enzyme has been less extensively studied, but in contrast to the pro-survival actions of SK1, this enzyme has been shown to enhance apoptosis via activation of caspase-3 [Liu *et al.*, 2003]. Induction of cell death by this enzyme was reported to be independent of S1P receptor activation but the presence of the Bcl-2 homology 3 (BH3) domain within SK2 was required. The BH3 domain is a nine amino acid sequence present in the group of “BH3 domain only” proteins, which are pro-apoptotic members of the Bcl-2 family. Interestingly, the SK1 enzyme does not contain a BH3 domain and this

difference may explain the opposing effects of these enzyme isoforms on cell fate.

Cellular effects of S1P may be divided into two broad categories: growth related functions or cytoskeletal functions. Effects on growth are often cell type dependent and include inhibition or stimulation of proliferation, altered differentiation, inhibition or activation of apoptosis and promotion of cell survival. Cytoskeletal S1P effects include chemotaxis, changes in cell morphology and aggregation. These varied responses are in part controlled by S1P receptor activation and the fate of a given cell is likely to depend on the profile of S1P receptors expressed and the presence or absence of costimulators such as PDGF.

Given its importance in regulation of cell signalling, S1P is liable to be involved in the pathology of many disease states. As was demonstrated by deletion of the S1P₁ gene in mice [Liu *et al.*, 2000], S1P is a key molecule in the progression of angiogenesis. This process is likely to be of key importance in tumour growth and other diseases where blood vessel formation is important, such as rheumatoid arthritis, diabetic retinopathy and psoriasis [reviewed by Pyne and Pyne, 2000a; Toman *et al.*, 2001]. S1P promotes cell survival and motility and may therefore be involved in tumour proliferation and invasion. Since S1P affects neuronal cells to promote survival, it has been suggested that the role of S1P in neurological disease states which are associated with deregulated apoptosis, such as Alzheimer's and Parkinson's diseases should be examined [Pyne and Pyne, 2000a].

The presence of S1P in serum and its release from blood-borne cells suggests that this molecule may be a vasoactive and cardioactive mediator. Indeed, the S1P₁ receptor was identified as an abundant transcript induced in endothelial cell differentiation [Hla and Maciag, 1990] and S1P₁ and related receptors are expressed in cardiovascular tissues. There is a role for S1P in cardiac development, as demonstrated by the phenotype of the zebrafish *Mil* knockout [Kupperman *et al.*, 2000] and the observation that S1P activation of S1P₁ promotes activation of eNOS and production of the potent vasodilator NO [Igarashi and Michel, 2001]. S1P induces well defined effects in vascular smooth muscle cells, particularly chemotaxis and proliferation, that are mediated by the S1P₁ receptor [Hla, 2003].

S1P may also function as an immune system regulator. This lipid is important in allergic responses and the balance between sphingosine and S1P was shown to be decisive for mast cell activation after triggering of the immunoglobulin (Ig) E receptor [Prieschl *et al.*, 1999]. This effect reflects activation of SK activity in response to IgE receptor activation. S1P secretion into the lung is increased after allergen challenge and mediates airway smooth muscle cell contraction and proliferation which leads to the characteristic hypercontractility and remodelling seen in asthma (reviewed by Jolly *et al.*, [2002]). Graeler and Goetzl, [2002] reported that ligation of the T cell receptor on resting T cells regulates the expression of S1P₁ and S1P₄ receptors. This suggests that S1P may modulate the function of naïve and activated T cells. The mode of action of the sphingosine analogue immunomodulatory drug, FTY720 implies a role of S1P as a regulator of immune cell trafficking (discussed further in this chapter, section 5).

Of the numerous reports describing the effects of S1P on a given cell type, those concerning cells which express the S1P₄ receptor are of particular interest since they may suggest a role for this receptor. Fueller *et al.*, [2003] observed S1P-induced mobilisation of intracellular calcium in human monocytes and macrophages. These cells express mRNA for S1P_{1,2,4} receptors although it is not clear which of these receptors accounts for this response. The effects of S1P on migration of murine splenic T cells have also been investigated [Graeler and Goetzl, 2002]. Resting T cells express S1P₁ and S1P₄ receptors and activation of these receptors was proposed to account for the observed stimulation of T cell chemotaxis by S1P. Activated T cells did not respond to S1P in this manner and this was thought to be a consequence of the marked decrease in expression of S1P_{1/4} receptors after T cell receptor activation. The effects of S1P on T cell mobility were believed to be mediated via the S1P₁ receptor, since transfection of this receptor into the HTC4 cell line (which does not respond to S1P) enhanced chemokinesis of these cells, whilst expression of recombinant S1P₄ did not. Another recent study described the ability of S1P to dose-dependently inhibit T cell proliferation [Jin *et al.*, 2003]. S1P has also been shown to stimulate chemotaxis of natural killer (NK) cells. The receptor which mediated this effect was not identified, however sensitivity of this response to pertussis toxin suggests involvement of S1P₁ [Kveberg *et al.*, 2002]. In contrast to the effects of chemokines, which recruit and activate NK cells, S1P recruits NK cells but inhibits their cytolytic activity against tumour cells [Maghazachi, 2003]. The effects of S1P on cells of the immune system suggest that this molecule is an important immunoregulator, which acts to maintain immune system homeostasis.

In contrast to the clear role of S1P as an activator of specific cell-surface GPCRs, evidence to support the concept that S1P is a second messenger is less clear-cut (reviewed by [Pyne and Pyne, 2000b; Hla, 2003; Young and Nahorski, 2002; Spiegel and Milstien, 2003]). The idea that S1P was a second messenger arose from its ability to mobilise calcium from intracellular stores in a manner that was dependent on the activation of sphingosine kinase. Many cell stimuli elicit S1P production, although it is likely that many of the functions previously attributed to the “second messenger” actions of S1P actually reflect activation of Edg family receptors (discussed by Pyne and Pyne [2000b]). Much of the evidence supporting an intracellular role for S1P arose from agonist stimulation of SK and the use of SK inhibitors in mammalian systems. In support of the proposed dual effects of S1P, dhS1P, which binds to and activates S1P receptors with comparable affinity and potency to S1P itself, does not mimic all the effects of S1P, particularly those related to cell survival (reviewed by Pyne and Pyne [2000b]).

Calcium mobilisation occurs after stimulation of HEK293 cells with S1P. However, this response was found to be independent of PLC activation and correlated with activation of SK and the transient production of S1P [Meyer zu Heringdorf *et al.*, 2001]. This suggested the existence of an alternative pathway for calcium mobilisation in mammalian cells. It has been proposed that intracellular S1P has a general role in calcium mobilisation for many GPCRs since other GPCRs have been shown to mobilise calcium in a manner which was dependent on SK activation [Meyer zu Heringdorf *et al.*, 1998]. Although several other reports support this idea, the intracellular target of S1P, which is likely to be located in the endoplasmic reticulum, has not yet been identified.

Sphingosine kinase orthologues exist in lower organisms that do not express lipid-responsive GPCRs. In yeast, deletion of the two SK genes was associated with decreased survival after heat stress [Hla, 2003]. S1P is also produced in plants and regulation of stomatal function was found to be dependent on SK activation [Coursol *et al.*, 2003]. These responses in yeast and plants, which occur after environmental stress, also involve calcium regulation, suggesting that S1P might have a universal role in calcium regulation [Spiegel and Milstien, 2003]. This is an interesting concept since calcium itself has been suggested as a regulator of SK activity [Pyne and Pyne, 2000b]. Identification of the intracellular S1P targets in plants or yeast will be valuable in understanding the role of S1P as a second messenger in mammalian cells.

S1P is a bioactive lipid, which mediates myriad effects in diverse cell types. These effects are influenced by the repertoire of cell-surface-expressed S1P receptors and the presence or absence of co-stimulatory signals. It seems likely that the site of action of intracellular S1P will be identified, confirming S1P as an extracellular agonist of S1P receptors and an intracellular second messenger involved in calcium mobilisation. This is not unprecedented since the lipid mediators leukotriene B₄ (LTB₄) and LPA have been shown to activate both cell-surface and nuclear receptors [Yokomizo *et al.*, 1997; Gobeil *et al.*, 2003].

1.4.3 Phytosphingosine-1-phosphate

The related compound, phS1P (*D-ribo*-phytosphingosine-1-phosphate) shown in Figure 1.11, was recently found to be a high affinity ligand for the S1P₄ receptor [Rios Candelore *et al.*, 2002]. In phS1P, the double bond present in S1P has been reduced to yield an extra hydroxyl group on the C4 carbon atom.

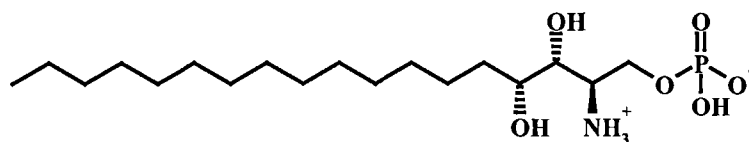


Figure 1. 11
Structure of phS1P.

Although the exact route of synthesis of phS1P has not been shown *in vivo*, the likely precursor (phytosphingosine) is produced in mammalian cells by the recently identified alkaline ceramidase aPHC [Mao *et al.*, 2001]. The activity of certain alkaline and neutral ceramidases may be regulated by growth factors and cytokines, which is suggestive of a role in mediation of cell signalling. It is probable that phytosphingosine is a substrate for the sphingosine kinase enzymes and it is through this route that phS1P is produced. Traditionally, phytoceramide (and phS1P) have been thought of as only being present in high concentrations in lower eukaryotes, such as *Sacchromyces cerevisiae*, however the recent detection of trace amounts of phytoceramide in mammalian tissues such as skin, liver and kidney coupled with the expression of aPHC mRNA in these tissues suggests that phS1P may have an important physiological role to play in these environments [Mao *et al.*, 2001]. These researchers suggested that the relevance of phS1P as a ligand for S1P₄ might be linked with the restricted expression pattern of aPHC. The presence of aPHC mRNA and its product (phytosphingosine) in skin has been highlighted as particularly interesting since Langerhans cells are skin-specific dendritic cells which strongly express S1P₄.

PhS1P binds to human S1P₄ with a K_d value of approximately 2 nM and is displaced by competition with dhS1P, S1P and FTY720-P, suggesting a shared binding pocket. The ability of phS1P to act as an agonist of S1P₄ was shown

using microphysiometer analysis. In this system, S1P was defined as a full agonist and had an EC_{50} of approximately 12 nM, whilst phS1P was a partial agonist with an EC_{50} of approximately 30 nM.

The physiological relevance of phS1P has yet to be investigated and the possibility that this compound represents the true *in vivo* ligand for S1P₄ is speculation. It has not been reported whether phS1P is a high affinity ligand solely for S1P₄, or whether it can bind strongly to the other S1P receptors, although given the structural similarity with S1P, it seems likely that phS1P would bind to other S1P receptors.

1.5 FTY720 and S1P receptors

Immunosuppressant drugs, such as cyclosporin A (CsA), are used in the prevention of organ transplant rejection and for the treatment of autoimmune diseases including rheumatoid arthritis and psoriasis. The mode of action of this class of compound is to suppress immune responses by inhibiting production of the cytokine interleukin-2 (IL-2) by antigen-stimulated T cells. However, their use is associated with severe side effects, including hepatic and renal toxicity.

Recently, the compound FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-propane-1,3-diol) has been identified as a potent immunomodulatory compound (shown in Figure 1.12). This compound has a highly novel mechanism of action and is believed to exert its effects via activation of S1P receptors. FTY720 is a synthetic structural analogue of a fungal metabolite derived from *Isaria sinclairii*, which has been used as a traditional Chinese herbal medicine for thousands of years and is considered as one of the three oriental medicines that bestow eternal youth [Im, 2003]. The development of FTY720 is a classic

example of ethnopharmacology, wherein the biological mechanism for a traditional therapeutic herbal agent has been elucidated and has resulted in the chemical optimisation of a novel class of compound.

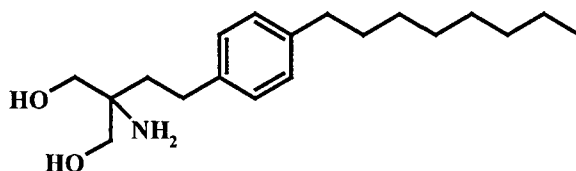


Figure 1. 12
Structure of FTY720.

FTY720 functions *in vivo* to promote reversible lymphopenia via retention of circulating B and T cells in secondary lymphoid organs [Chiba *et al.*, 1998]. The effect of T cell redistribution is to reduce the numbers of these cells at sites of inflammation and hence to attenuate the immune response. The compound has been extensively profiled in animal transplantation models, including skin, islet and solid organ allograft (reviewed by Brinkmann and Lynch [2002]) and synergises with traditional immunosuppressive agents, allowing combination therapy of FTY720 with subclinical doses of compounds such as CsA. FTY720 monotherapy in models of adjuvant- and collagen-induced arthritis and systemic lupus erythmatosus has also been successfully applied. Low doses of FTY720 can reportedly be used as a prophylactic or a therapeutic in the model of multiple sclerosis, experimental autoimmune encephalomyelitis. Efficacy of this compound has been shown in human phase II trials for the prevention of acute rejection in *de novo* renal patients on baseline immunosuppression [Brinkmann and Lynch, 2002].

Administration of FTY720 promotes sequestration of naïve and activated CD4 and CD8 T cells and B cells from the circulation into lymph nodes and Peyer's patches [Xie *et al.*, 2003]. Although the effects of this compound had been known for some years, its mode of action was unclear. Sensitivity of the lymphocyte homing effect to pertussis toxin suggested the involvement of a $G\alpha_i$ coupled GPCR and the chemokine receptor CCR7 was proposed as a potential candidate. CCR7 and its chemokine ligands, ELC (EBV-induced molecule 1 ligand chemokine) and SLC (secondary lymphoid tissue chemokine) are known to play a critical role in the migration of B and T cells into secondary lymphoid organs. It was subsequently shown that FTY720 did not act via this chemokine receptor, since the compound was still able to promote lymphopenia in both CCR7 deficient and *plt* mice, which do not express either ligand for CCR7 [Henning *et al.*, 2001], although the kinetics of FTY720-induced lymphocyte homing in these mice were delayed compared to wild type control animals.

The breakthrough in determining the way in which FTY720 elicits its response came with the observation that FTY720 is rapidly metabolised *in vivo* by addition of phosphate to the terminal alcohol group to yield the compound FTY720-P (shown in Figure 1.13). The structure of this metabolite bears noticeable resemblance to that of S1P and it was proposed that FTY720-P may act at S1P receptors. Two independent groups recently identified this compound as a high affinity (K_d 's 0.2-6 nM), potent agonist at four of the five S1P receptors (S1P_{1,3,4,5}) [Mandala *et al.*, 2002; Brinkmann *et al.*, 2002], but not the S1P₂ receptor. FTY720 was shown to be rapidly converted *in vivo* to the phosphate, and this conversion could also be mediated via the isolated sphingosine kinase enzyme, albeit more slowly. Since T cells express mRNA for S1P_{1,4,5} receptors,

the implication of these studies was that activation of one or more S1P receptors present on T cells promoted sequestration of circulating T cells to secondary lymphoid organs. FTY720 is the first example of the application of a sphingomimetic compound as a therapeutic agent (reviewed by Kester and Kolesnick [2003]).

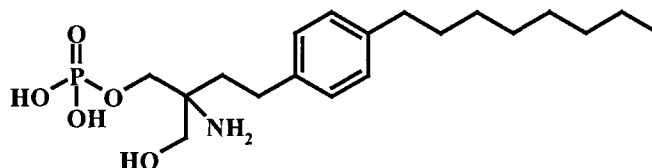


Figure 1. 13
Structure of FTY720-P.

In a subsequent study, the effect of FTY720 on T cell chemotaxis *in vitro* was investigated [Graeler and Goetzl, 2002]. FTY720 did not stimulate movement of murine T cells when tested at concentrations of 1-100 nM, but could dose-dependently inhibit S1P-induced T cell chemotaxis *in vitro*. These results were interpreted as suggesting that FTY720 has no direct chemotactic activity but can suppress the stimulatory effects of S1P on T cell migration. The apparent contradiction between these results and the previous findings that the lymphopenic action of FTY720 was a consequence of its actions as a high affinity S1P receptor agonist highlight the need for further research in this area.

The report of Brinkmann *et al.*, [2002] identified the phosphorylated form of the (R)-isomer of the related compound, AAL-P (2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol) [Figure 1.14] to be another potent agonist of S1P_{1,3,4,5} receptors. This compound was a more potent agonist of S1P₄ than FTY720-P ($EC_{50} = 4$ nM compared to 63 nM for FTY720-P [Brinkmann and Lynch, 2002]). The

unphosphorylated AAL compound exhibited similar *in vivo* lymphopenic effects to those of FTY720.

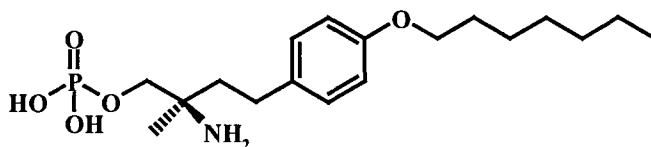


Figure 1. 14
Structure of (R)-AAL-P.

Although it seems highly likely that the lymphopenic effect of FTY720 administration occurs, at least in part, as a result of activation of S1P receptors expressed on T cells, the precise manner in which the T cells are recruited to, and held in lymph nodes and Peyer's patches is unclear. Recent advances have suggested that FTY720 has dual effects to promote increased intrinsic mobility of peripheral B and T cells and to increase endothelial integrity, thereby preventing egress from secondary lymphoid organs.

These exciting observations have significantly increased interest in the S1P receptors as potential therapeutic targets and have also identified highly potent agonists against four of the five receptors. The actions of FTY720-P have suggested an important role for S1P in lymphocyte trafficking and control of the immune response.

1.6 Project aims

When the work described in this thesis was started, S1P₄ was the orphan receptor named Edg6. From sequence similarities with other Edg receptors, it seemed likely that the Edg6 orphan would be activated by the lysophospholipid S1P and would couple to Gα_i G proteins. Therefore, the first aim of this work was to

express S1P₄ in mammalian cells and investigate whether the receptor coupled to this class of G proteins and could be activated by S1P. It was known that other members of the S1P and LPA receptor families were widely expressed in mammalian cells and that their presence could lead to the production of interfering signals in functional assays. A fusion protein between S1P₄ and a mutated, pertussis toxin-insensitive variant of the G α_{i1} G protein was also used in these investigations so that potential interference due to activation of endogenous Edg receptors present in host cells could be eliminated via treatment with pertussis toxin.

Since it was expected that the host cells used for expression of the recombinant S1P₄ and S1P₄-G α_{i1} fusion proteins would express endogenous Edg family receptors, the second aim of this work was to use the technique of RT-PCR to determine the profile of LPA and S1P receptors endogenously expressed within these cells.

Many GPCRs exhibit the phenomenon of constitutive activity, and results generated in the early stages of this thesis using a [³⁵S]GTP γ S binding assay to detect S1P₄ activation suggested this receptor to be constitutively active. At the time of these investigations, no inverse agonists were available for this class of GPCRs and so it was necessary to use an alternative approach to further characterise agonist-independent signalling by this receptor. Therefore, the relationship between the expression level of S1P₄ and the level of basal signalling was investigated using an mammalian inducible expression system.

As would be expected, many laboratories are working on the S1P receptor family and significant findings were reported during the course of the investigations

described in this thesis. As a consequence, the aims of this project developed as new observations concerning this receptor family were published. The final aim of the work presented in this thesis was to examine the nature of ligand preference exhibited S1P₄, in the light of published observations. Firstly, the activity of newly identified agonists such as phS1P was determined using the [³⁵S]GTPγS binding assay. Novel information regarding the role of the conserved E¹²¹ residue present in the S1P receptor family was then used to further understand the nature of S1P₄ ligand preference. The role of this residue in S1P receptors has been investigated using the S1P₁ receptor to exemplify this receptor family. Mutation of this residue to glutamine (present at this position in the LPA receptor family) was found to influence ligand selectivity and allowed activation of S1P₁ by LPA, whilst preventing its response to S1P [Wang *et al.*, 2001]. The second part of this final aim was to further test the hypothesis that the conserved E¹²¹ residue of S1P receptors is key in controlling ligand selectivity. This was achieved by introduction of the equivalent mutation in the S1P₄ receptor and evaluation of its consequences on activation of [³⁵S]GTPγS binding by S1P and LPA after expression of the mutant receptor in mammalian cells.

Chapter 2 – Materials and Methods

2.1 General chemicals and materials

General laboratory chemicals were purchased from Sigma Aldrich Company Ltd., (Gillingham, Dorset, U.K.) or BDH Ltd., (Poole, Dorset, U.K.) unless stated otherwise. Ethanol and Biphenol/Tris were from Hayman Ltd., (Witham, Essex, U.K.) and Camlab Ltd., (Cambridge, U.K.), respectively. Bacto-tryptone, bacto-agar and bacto-yeast extract were from Difco Ltd., (Detroit, MI., U.S.A.).

Restriction endonucleases, dNTPs, DNA molecular weight markers, T4 DNA ligase, calf intestinal phosphatase and amplification grade deoxyribonuclease I were purchased from New England Biolabs Ltd., (Hitchin, Herts., U.K.) or Roche Diagnostics Ltd., (Lewes, East Sussex, U.K.). DNA sequences were cloned into pCR4-TOPO, pIRESpuro or pIND expression vectors, Invitrogen Ltd., (Paisley, Scotland, U.K.). QIAGEN Miniprep, Maxiprep, QIAquick Gel Extraction, PCR purification, DNeasy genomic DNA and RNeasy Mini RNA purification kits were from Qiagen Ltd., (Crawley, West Sussex, U.K.). Apparatus for horizontal gel electrophoresis was supplied by Invitrogen Ltd., (Paisley, Scotland, U.K.).

Big Dye Terminator Cycle Sequencing Ready Reaction DNA sequence kit and Amplitaq DNA polymerase were obtained from Applied Biosystems, (Warrington, Cheshire, U.K.). Sequencing reactions were carried out using a Biometra Trio-Thermoblock (Anachem Ltd., Luton, Herts., U.K.) or Primus 96 Plus (MWG-Biotech Ltd., Milton Keynes, Bucks., U.K.). All other PCR reactions were performed using Biometra Personal Cyclor (Anachem Ltd., Luton, Herts., U.K.) or Primus 96 Plus (MWG-Biotech Ltd., Milton Keynes,

Bucks., U.K.) PCR machines. Advantage RT-for-PCR, Advantage 2 and Advantage-GC 2 PCR kits were from Clontech Laboratories Inc., (Palo Alto, CA, U.S.A.).

Media and supplements for tissue culture and transfection reagents were supplied by Invitrogen Ltd., (Paisley, Scotland, U.K.); non-enzymatic cell dissociation solution was from Sigma Aldrich Company Ltd., (Gillingham, Dorset, U.K.); foetal bovine serum was from Helena Biosciences Ltd., (Sunderland, U.K.) or PAA Labs GmbH., (Linz, Austria) and tissue culture plastics were from BD Falcon (Erembodegem, Belgium). CHO-K1 and HEK-293 cells were from ECACC (CAMR, Porton Down, Wilts., U.K.) and CHO-EcR cells were supplied by Invitrogen Ltd., (Paisley, Scotland, U.K.).

Sphingosine-1-phosphate was obtained from Biomol Research Laboratories Inc., (Plymouth Meeting, PA., U.S.A.) and UK14304 was from Tocris Cookson Ltd., (Bristol, U.K.). Pertussis toxin was purchased from CN Biosciences Ltd., (Nottingham, U.K.). Lysophosphatidic acid (18:1, 16:0 and 14:0) was from Avanti Polar Lipids Inc., (Alabaster, AL., U.S.A.). Other chemicals (FTY720 and AAL) were synthesised by the Medicinal Chemistry department of Celltech R&D Ltd.

The radiochemical guanosine 5'-[γ - ^{35}S]triphosphate, triethylammonium salt ([^{35}S]GTP γ S) had a specific activity of $> 37 \text{ TBq/mmol}$ and was purchased from Amersham Biosciences (Amersham, Bucks., U.K.).

Tris-Glycine SDS Page gels and SeeBlue prestained protein markers were supplied by Invitrogen Ltd., (Paisley, Scotland, U.K.). Immobilon-P PVDF membrane was from Millipore Ltd., (Watford, Herts., U.K.).

Small scale centrifugation was performed using Eppendorf 5415D (Anachem Ltd., Luton, Herts., U.K.) or Hettich refrigerated EBA12R (Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany) benchtop microcentrifuges; Sorvall RC3B or RC5B refrigerated centrifuges were used for large scale centrifugation.

2.1.1 Media for bacterial growth

LB Agar	1 % (w/v) Bacto-tryptone 0.5 % Bacto-yeast extract 171 mM NaCl 1.5 % (w/v) Bacto-agar pH 7.0
SOC	2 % (w/v) Bacto-tryptone 0.5 % Bacto-yeast extract 8.6 mM NaCl 25 mM KCl 10 mM MgCl ₂ 20 mM glucose pH 7.0
2x TY Broth	2 % (w/v) Bacto-tryptone 1 % Bacto-yeast extract 171 mM NaCl 1.5 % (w/v) Bacto-agar pH 7.0

2.1.2 Buffer formulations

PBS	137 mM NaCl 2.68 mM KCl 10.1 mM Na ₂ HPO ₄ 1.76 mM KH ₂ PO ₄ pH 7.4
TAE	40 mM Tris-acetate pH 8.0 2.5 mM EDTA

TE	10 mM Tris-HCl pH 7.4 1 mM EDTA
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Hi TNE	65 mM Tris-HCl pH8 15 mM EDTA 4 M NaCl
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5x DNA loading buffer

0.25 % (w/v) bromophenol blue, 30 % (w/v) glycerol

2x protein sample buffer

125 mM Tris-HCl pH 6.8, 0.715 M 2-mercaptoethanol, 20 % (w/v) glycerol,
4 % (w/v) SDS, 0.1 % (w/v) bromophenol blue

5x ligation buffer

250 mM Tris-HCl pH 7.4, 5 mM ATP, 100 mM DTT, 50 mM MgCl₂

Clontech Advantage PCR and RT buffers

10x Advantage 2 PCR buffer	400 mM Tricine-KOH, pH 9.2 at 25 °C 150 mM potassium acetate 35 mM magnesium acetate 37.5 µg/mL BSA 0.05 % (v/v) Nonidet P-40 0.05 % Tween-20
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5x Advantage-GC 2 PCR buffer	200 mM Tricine-KOH, pH 9.2 at 25 °C 75 mM potassium acetate 17.5 mM magnesium acetate 25 % (v/v) DMSO 18.75 µg/mL BSA 0.025 % (v/v) Nonidet P-40 0.025 % Tween-20
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5x Advantage RT-for-PCR buffer	200 mM Tris-HCl, pH 8.3 375 mM KCl 15 mM MgCl ₂
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QIAGEN kit purification buffers

Buffer AE (elution buffer)	*
Buffer AL (lysis buffer)	*
Buffer AW1 (wash buffer 1)	*
Buffer AW2 (wash buffer 2)	*
Buffer EB (elution buffer)	10 mM Tris-HCl, pH8.5
Buffer N3 (neutralisation buffer)	Contains chaotropic salts *
Buffer P1 (resuspension buffer)	50 mM Tris-HCl, pH 8.0 10 mM EDTA 100 µg/mL RNase A
Buffer P2 (lysis buffer)	200 mM NaOH 1 % (w/v) SDS
Buffer P3 (neutralisation buffer)	3.0 M potassium acetate, pH 5.5
Buffer PB (binding buffer)	Contains chaotropic salts *
Buffer PE (wash buffer)	*
Buffer QBT (equilibration buffer)	750 mM NaCl 50 mM MOPS, pH7.0 15 % (v/v) isopropanol 0.15 % (v/v) Triton X-100
Buffer QC (wash buffer)	1.0 M NaCl 50 mM MOPS, pH7.0 15 % (v/v) isopropanol
Buffer QF (elution buffer)	1.25 M NaCl 50 mM Tris-HCl, pH 8.5 15 % (v/v) isopropanol
Buffer QG (solubilisation buffer)	*
Buffer RLT (lysis buffer)	*
Buffer RW1 (wash buffer 1)	*
Buffer RPE (wash buffer 2)	*

* The composition of these QIAGEN buffers is withheld by the manufacturer.

2.1.3 Sources of cDNA

The human peripheral blood mononuclear (PBMC) cDNA library had been previously prepared at Celltech R&D Ltd. $G\alpha_{i1}(C^{351}I)$ and $\alpha_{2A}AR-G\alpha_{i1}(C^{351}I)$ constructs were kindly provided by Professor Graeme Milligan (University of Glasgow, Scotland, U.K.).

2.1.4 Bacterial strains

Routine propagation of recombinant plasmids was performed using Novablue chemically competent *E. Coli* cells (*endA1 hsdR17(r_{K12}⁻ m_{K12}⁺) supE44 thi-1 recA1 gyrA96 relA1 lacF⁺[proA⁺B⁺ lacI^qZ M15::Tn10 (Tc^R)*]), supplied by Novagen Inc., (Madison, WI., U.S.A.). TOPO cloning reactions were transformed into TOP10 chemically competent *E. Coli* cells (*F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG*), supplied by Invitrogen Ltd., (Paisley, Scotland, U.K.).

2.1.5 Antibodies

High affinity rat anti-HA monoclonal antibodies (unconjugated and Fluorescein-conjugated), which recognises the HA epitope tag (YPYDVPDYA) were from Roche Diagnostics Ltd. Rabbit polyclonal anti- $G\alpha_{i1}$ (sc-391) and anti $G\alpha_{i1/2/3}$ (sc-262) antibodies were supplied by Autogen Bioclear Ltd., (Calne, Wilts., U.K.). The rabbit polyclonal SG1 antibody was raised against the decapeptide KENLKDCGLF [Grant *et al.*, 1997] and cross-reacts with $G\alpha_{i1/2}$. Peroxidase conjugated anti-rat and anti-rabbit IgG antibodies were purchased from Jackson ImmunoResearch Laboratories Inc., (West Grove, PA., U.S.A.). Isotype matched negative control antibody for use with the rat anti-HA-Fluorescein

antibody was FITC conjugated rat IgG1, κ and was supplied by BD Pharmingen (Erembodegem, Belgium). The anti-S1P₄ antibody was a rabbit polyclonal antibody that was raised against a peptide corresponding to amino acids 226-243 of the human S1P₄ sequence (Lifespan Biosciences, Inc., Seattle, WA., U.S.A.).

2.1.6 Oligonucleotides

Oligonucleotides were designed using sequences in the Genbank nucleotide sequence database. Shown below are sequences for oligonucleotides that were used for PCR cloning, PCR mutagenesis and RT-PCR; vector specific and sequencing oligonucleotides are not shown.

Human S1P₄ (Edg6) – Genbank accession number AJ000479

F7240 (forward)

5'-GAGAGAGCGGGCCGCCACCATGAACGCCACGGGGACC-3'
 NotI Start

F7241 (reverse)

5'-GAGAGAGAATTCTTATCAGATGCTCCGCACGCTGGAG-3'
 EcoRI Stop Stop

F12582 (forward)

5'-GAGAGAGCGGGCCGCCACCATGTATCCATATGATGTTCCAGATTAT
 NotI Start HA-tag
GCTAACGCCACGGGGACCCCGGTG-3'

F12581 (reverse)

5'-GAGAGACCATGGTGGCAAAGCGCTCCCC-3'
 NcoI

F27384 (forward)

5'-GAGAGAGGATCCTGGCCCTGGCCGTC-3'
 BamHI

F36663 (reverse)

5'-GAGAGAGAATTCGGCGATGCTCCGCACGCTGGAGATG-3'
 EcoRI A(aa)

110A (forward)

5'-GAGAGAGAAATTCGCCACCATGTATCCATATGATG-3'
 EcoRI Start

111A (reverse)

5'-GAGAGAGAGGCGGCCGCTTATCAGATGCTCCGCACGCTG-3'
 NotI Stop Stop

58A (forward)

5'-CAGTGGTTCCTACGGCAGGGCCTGCTCTTCAC-3'
 E¹²²Q

59A (reverse)

5'-GTGAAGAGCAGGCCCTGCCGTAGGAACCACTG-3'
 E¹²²Q

Gα_{i1} – Genbank accession number M17527

Oligonucleotides were designed to allow PCR amplification of Gα_{i1}, which had previously been modified to contain the C³⁵¹I mutation, which confers insensitivity to pertussis toxin [Bahia *et al.*, 1998]; the presence of this mutation affected design of the reverse primer and its location in this primer is shown.

F36661 (forward)

5'-GAGAGAGAAATTCGCCACCATGGGCTGCACACTGAGCG-3'
 EcoRI Start

F36662 (reverse)

5'-GAGAGAGGATCCTTAGAAGAGACCGATGTCTTTTAG-3'
 BamHI Stop C³⁵¹I

Cloning of partial sequences of Edg receptor genes from Chinese hamster genomic DNA was achieved using the following oligonucleotide pairs, which had been designed using alignments of nucleotide and protein sequences from available species. The suffix B indicates oligonucleotides which were used for

cloning fragments of Chinese hamster Edg receptor genes; those designated with the suffix C were used for RT-PCR.

Chinese hamster S1P₁ (Edg1)

1170B (forward)

5'-GCTGGGTCATCTCCCTCATCCTGG-3'

1171B (reverse)

5'-GGTCAGAGTGTAGATGATGGGGTTG-3'

1364C (forward)

5'-CCATCATGGGCTGGAACTGCATC-3'

1365C (reverse)

5'-ACCTGAGTTCAGCACAGCCAGAA-3'

Chinese hamster S1P₂ (Edg5)

1176B (forward)

5'-AACAGCAAGTTCCACTCAGCAATGTA-3'

1177B (reverse)

5'-GCAGCCAGCAGAYGATRAARACRCC-3'

1372C (forward)

5'-CTGTTCTCTCGGCAACCTGGCAG-3'

1373C (reverse)

5'-CAGTACGATGGTGACTGTCTTGAG-3'

Chinese hamster S1P₃ (Edg3)

1215B (forward)

5'-CTGATGGTTTTGATTGCCATCTGGA-3'

1217B (reverse)

5'-CCAGCGTGTAGATGACNNGGGTTCA-3'

1368C (forward)

5'-AACATAAATTTCACAACCGCATGTAC-3'

1369C (reverse)

5'-GATGAACACACTCACTACTATCACG-3'

Chinese hamster S1P₄ (Edg6)

53B (forward)

5'-GAGAGTGGGGCCACCAAGACCAGCCG-3'

54B (reverse)

5'-CCTCACGGCTGCGGAAGGAGTAGATGA-3'

1374C (forward)

5'-GTGTATGGTTGCATCGGGCTGTG-3'

1375C (reverse)

5'-GGGTTGATGGCAGAGTTGAGTACG-3'

Chinese hamster S1P₅ (Edg8)

1182B (forward)

5'-GGTTTGCGCCGNGARGGRGGYGTC-3'

58B (reverse)

5'-GTCCGGCAGGTCKCGGTTGGTGA-3'

1378C (forward)

5'-GTGGCACTGGCCGCATCGGTG-3'

1379C (reverse)

5'-GAACGTGTAGATGATGGGATTCAG-3'

Chinese hamster LPA₁ (Edg2)

45B (forward)

5'-CACAGCCCCAGTTCACAGCCATG-3'

46B (reverse)

5'-GTTGAAAATGGCCCAGAAGACTAAG-3'

1366C (forward)

5'-CGAACCCCAGTGCTTCTACAACG-3'

1367C (reverse)

5'-GTAGGAGTCACTATAGAGGGGTG-3'

Chinese hamster LPA₂ (Edg4)

49B (forward)

5'-GGCCAGTGCTACTACAACGAGACCA-3'

1174B (reverse)

5'-AGACAAGCAGGCTSGAYAGRGCCCA-3'

1370C (forward)

5'-ATTGGCTTTTTCTACAATAACAGTGG-3'

1371C (reverse)

5'-CACAGCCAGGTATGAGCGGCTG-3'

Chinese hamster LPA₃ (Edg7)

1178B (forward)

5'-GAGTGTCCTACTATGACAAGCNCATGG-3'

1180B (reverse)

5'-CAGTCATCACYGTCTTCATTAGCTTCA-3'

1376C (forward)

5'-GACTTTTTTTACAACAGGAGCAACTC-3'

1377C (reverse)

5'-AGCCCTCCGGCGGCTGATGGA-3'

Chinese hamster GAPDH – Genbank accession number X52123

59C (forward)

5'-GGTCGGCGTGAACGGATTTGGCCG-3'

60C (reverse)

5'-CGCATTGCTGACAATCTTGAGGGAG-3'

2.1.7 Mammalian cell lines

CHO-K1 cells (85051005) and HEK-293 cells (85120602) were from ECACC (CAMR, Porton Down, Wilts., U.K.) and CHO-EcR cells were supplied by Invitrogen Ltd., (Paisley, Scotland, U.K.).

2.2 DNA methods

2.2.1 PCR amplification of DNA fragments

Due to its high GC content, amplification of S1P₄ sequences was performed using Advantage-GC 2 PCR kit. Typically, PCR reactions were carried out in a total volume of 20 μ L and contained approximately 100 ng cDNA, genomic DNA or plasmid DNA template, 10 pmoles each oligonucleotide, 4 μ L Advantage-GC 2 PCR buffer, 0.5 M GC Melt, 0.2 mM dNTP mix (dATP, dCTP, dGTP, dTTP), 1 μ L Advantage 2 Polymerase and sterile dH₂O.

Other PCR reactions were performed using the Advantage 2 PCR kit. Reactions were typically set up in a final volume of 20 μ L and comprised approximately 100 ng cDNA or plasmid DNA template, 10 pmoles each oligonucleotide, 2 μ L Advantage 2 PCR buffer, 0.2 mM dNTP mix (dATP, dCTP, dGTP, dTTP), 1 μ L Advantage 2 Polymerase and sterile dH₂O.

When PCR reactions were performed to generate DNA for use in cloning, reactions were scaled up to 50 μ L, with the components in the same proportions as described above for 20 μ L reactions.

Oligonucleotides were normally designed to have a melting temperature (T_m) of at least 68 °C.

Reactions were cycled under the following conditions:

“2 step program”

95 °C	1 minute] x35 cycles
95 °C	30 seconds	
68 °C	3 minutes	
68 °C	3 minutes	
4 °C	constant	

The two step PCR program was used for general PCR amplification and cloning activities. Amplification of partial Edg receptor sequences from Chinese hamster genomic DNA, and from first strand cDNA prepared from Chinese hamster RNA, used either this two step program, or the three step program shown below; details of which program was used for amplification of which partial sequence are shown in Table 2.1.

“3 step program”

94 °C	1 minute] x30 cycles
94 °C	15 seconds	
60 °C	30 seconds	
68 °C	1 minute	
68 °C	5 minutes	
4 °C	constant	

Post-reaction, samples were analysed using agarose gel electrophoresis.

	Forward oligonucleotide	Reverse oligonucleotide	PCR program
S1P ₁	1170B	1171B	2 step
	1364C	1365C	2 step
S1P ₂	1176B	1177B	3 step
	1372C	1373C	2 step
S1P ₃	1172B	1173B	3 step
	1368C	1369C	3 step
S1P ₄	53B	54B	2 step
	1374C	1375C	2 step
S1P ₅	1182B	58B	2 step
	1378C	1379C	3 step
LPA ₁	45B	46B	2 step
	1366C	1367C	2 step
LPA ₂	49B	1174B	3 step
	1370C	1371C	3 step
LPA ₃	1178B	1180B	3 step
	1376C	1377C	3 step

Table 2. 1

Oligonucleotides and PCR programs used to amplify S1P and LPA receptor gene fragments from Chinese hamster genomic DNA or first strand cDNA.

The suffix B denotes oligonucleotides used for cloning gene fragments from Chinese hamster genomic DNA; the suffix C denotes use in RT-PCR with first strand cDNA. Sequences of these oligonucleotides are shown in this chapter, section 1.6.

2.2.2 Analysis of DNA fragments by gel electrophoresis

DNA samples were analysed by horizontal electrophoresis, typically using 1 % (w/v) agarose gels in TAE buffer, which were run at 100 V/cm. Appropriate size markers were included on each gel. Ethidium bromide was added to both gel and running buffer, to a final concentration of 30 µg/mL. DNA bands were visualised using uv transillumination at 254 nm. Agarose gel electrophoresis was also used for the purification of DNA fragments for cloning; this was performed in the same manner as analytical gel electrophoresis.

2.2.3 Spin column purification of PCR products

Purification of PCR products which were to be ligated into expression vectors (after digestion with appropriate restriction enzymes) was performed using QIAGEN QIAquick PCR Purification Kit, in accordance with the manufacturer's protocol; all centrifugation steps were performed using a benchtop centrifuge at maximum speed (16,100 x g). Briefly, 5 volumes buffer PB were added to 1 volume of PCR reaction and mixed; the sample was then applied to a QIAquick spin column and centrifuged for 1 minute; the flow-through was discarded. Column-bound DNA was washed with 0.75 mL buffer PE and centrifuged for 1 minute, the flow-through discarded and the column centrifuged for a further minute. 30 µL buffer EB was applied to the centre of the column, which was allowed to stand for 1 minute at room temperature prior to centrifugation for 1 minute; this eluted the DNA solution.

2.2.4 Purification of DNA fragments from agarose gels

DNA fragments were purified from agarose gels using QIAGEN QIAquick Gel Extraction Kit, in accordance with the manufacturer's instructions; all centrifugation steps were performed using a benchtop centrifuge at maximum speed (16,100 x g). Briefly, the desired band was excised from the agarose gel and weighed; 3 volumes buffer QG were added per gel volume (and the assumption was made that 100 mg gel was approximately equal to 100 µL volume). The sample was incubated at 50 °C for 10 minutes in order to melt the agarose and mixed well; 1 volume isopropanol was mixed with the sample before it was applied to a QIAquick spin column and centrifuged for 1 minute. The flow-through was discarded and the column washed with 0.5 mL buffer QG

and centrifuged for 1 minute. Column-bound DNA was washed with 0.75 mL buffer PE and centrifuged for 1 minute, the flow-through discarded and the column centrifuged for a further minute. 30 μ L buffer EB was applied to the centre of the column and allowed to stand for 1 minute at room temperature prior to centrifugation for 1 minute; this eluted the DNA solution.

2.2.5 Analysis of DNA fragments by restriction enzyme digest

For general restriction enzyme analysis, 1-2 μ g DNA was incubated in an appropriate enzyme buffer with 5 units of each restriction enzyme for 2-4 hours at 37 °C. One unit is defined as the amount of enzyme required to completely digest 1 μ g of substrate DNA in 1 hour. When the DNA was to be used in subsequent sub-cloning procedures, the PCR product (which had been purified according to this chapter, sections 2.3-2.4), or approximately 10 μ g plasmid DNA was incubated with 20 units of each restriction enzyme, overnight at 37 °C; it was then subject to phenol-chloroform extraction prior to purification by gel extraction (this chapter, sections 2.4 and 2.7).

2.2.6 Alkaline phosphatase treatment of digested vector

Prior to ligation with DNA fragments, vector that had been digested with appropriate restriction enzymes was treated with alkaline phosphatase to prevent religation of partially digested vector. The reaction comprised approximately 10 μ g DNA, an appropriate volume of 10x phosphatase buffer and alkaline phosphatase (100 units). One unit is defined as the enzyme activity which hydrolyses 1 μ mol of 4-nitrophenyl phosphate in 1 minute at 37 °C. After incubation at 37 °C for 30 minutes, a further 100 units alkaline phosphatase were

added and the reaction incubated for another 30 minutes at 37 °C. The treated DNA was then purified by phenol-chloroform extraction (this chapter, section 2.7).

2.2.7 Phenol-chloroform extraction of DNA

After digestion with restriction enzymes or alkaline phosphatase treatment, DNA was subject to extraction with phenol/chloroform/isoamyl alcohol. Briefly, to a 100 µL reaction, 20 µL Hi TNE buffer, 80 µL TE buffer and 700 µL phenol/chloroform/isoamyl alcohol (25:24:1) were added and mixed well. Centrifugation (1 minute at 16,100 x g) was used to separate the aqueous and organic layers. The aqueous layer was carefully removed into a clean tube and the phenol/chloroform/isoamyl alcohol re-extracted after the addition of 20 µL Hi TNE buffer and 80 µL TE buffer. The aqueous layer was combined with the first aqueous fraction and extracted with chloroform. DNA in the resultant aqueous layer was then precipitated by the addition of 2.5 volumes of 100 % ethanol and incubation at -20 °C for 1 hour. The DNA was collected by centrifugation at 16,100 x g for 10 minutes, washed once with cold 70 % ethanol and resuspended in an appropriate volume of TE buffer.

2.2.8 Ligation of DNA fragments

Ligation of DNA fragments was performed in a total reaction volume of 20 µL, comprising 4 µL 5x ligation buffer, 400 units T4 DNA ligase, approximately 50 ng vector DNA and a molar equivalent of insert fragment(s). Control reactions, containing no insert DNA, were routinely performed to allow assessment of the background of uncut vector as well as the efficiency of

transformation. Ligation reactions were incubated overnight at 16 °C before transformation of a proportion of the reaction into competent *E. Coli* cells.

2.2.9 Transformation of DNA into competent *E. Coli* cells

Novablue competent *E. Coli* cells were transformed in accordance with manufacturer's recommendations. Briefly, 50 µL Novablue cells were gently thawed on ice and 1-2 ng DNA was added. The cells were incubated on ice for 5 minutes, heat-shocked at 42 °C for 30 seconds then incubated on ice for a further 2 minutes. 800 µL SOC medium was added and cells were incubated on a shaker platform at 37 °C for 30 minutes. An appropriate volume of sample was then plated out onto LB Agar plates supplemented with 100 µg/mL ampicillin, which were then grown overnight at 37 °C.

2.2.10 TOPO cloning of PCR products

PCR products which were amplified with the specific purpose of sequencing (i.e. partial sequences of S1P- and LPA-receptor coding sequences which were amplified from Chinese hamster genomic DNA) were cloned into pCR4-TOPO vector using the TOPO TA Cloning Kit for Sequencing, in accordance with the manufacturer's recommendations. Briefly, PCR products were purified from agarose as described in this chapter, section 2.4 and cloned into the TOPO TA vector in a 5 minute reaction. 2 µL of each reaction was then transformed into TOP10 One Shot chemically competent cells, which were incubated on ice for 5 minutes, heat shocked at 42 °C for 30 seconds and returned to ice. 250 µL SOC medium was added and the tube incubated on a shaker platform for 1 hour at 37 °C after which time appropriate volumes were plated out onto LB Agar

plates supplemented with 100 µg/mL ampicillin; these plates were then grown overnight at 37 °C.

2.2.11 Isolation of plasmid DNA using QIAGEN Miniprep columns

Miniprep columns were used to prepare recombinant plasmid DNA for subsequent analysis via restriction enzyme digest or DNA sequencing. The method used was broadly in accordance with the manufacturer's recommendations and centrifugation steps were performed using a benchtop centrifuge at maximum speed (16,100 x g), unless stated otherwise. Briefly, a single colony of transformed *E. Coli* was picked from a selective LB Agar plate and used to inoculate a 10 mL 2x TY culture (containing 100 µg/mL ampicillin). The culture was grown overnight at 37 °C and bacteria collected by centrifugation (1,500 x g for 15 minutes). The cell pellet was resuspended in 250 µL buffer P1, then 250 µL lysis buffer P2 was added and mixed by gentle inversion. The lysis reaction was neutralised by the addition of 350 µL buffer N3 and was immediately mixed by gentle inversion. Centrifugation (10 minutes) was used to clear the cell lysate and the supernatant fraction was applied to a QIAprep spin column. The column was placed in a 2 mL collection tube and centrifuged for 1 minute. Bound DNA was washed with 0.5 mL buffer PB then with 0.75 mL buffer PE; centrifugation for 1 minute followed each wash step. After an additional 1 minute centrifugation to remove any remaining PE buffer, DNA was eluted from the column in 180 µL buffer EB, which was collected by centrifugation, into a clean tube. The DNA was then precipitated by the addition of 20 µL Hi TNE buffer and 600 µL 100 % ethanol with incubation at -20 °C for

1 hour. Precipitated DNA was collected by centrifugation for 10 minutes, washed once with 70 % ethanol and resuspended in 30 μ L buffer EB.

2.2.12 Isolation of plasmid DNA using QIAGEN Maxiprep kit

Larger quantities of plasmid DNA were prepared from overnight cultures (200 mL 2x TY medium containing 100 μ g/mL ampicillin) using QIAGEN-tip 500, following the manufacturer's guidelines; centrifugation steps were performed using Sorvall RC5B refrigerated centrifuge. In brief, cells were collected by centrifugation (4,600 x g rpm for 15 minutes) and the cell pellet resuspended in 10 mL buffer P1. 10 mL buffer P2 was added and mixed gently to lyse the cells. After an incubation of 5 minutes at room temperature, 10 mL buffer P3 (which had been chilled to 4 °C) was added and the lysate was immediately poured into a QIAfilter cartridge and incubated for 10 minutes at room temperature. The cell lysate was then filtered through the QIAfilter cartridge and applied to a QIAGEN-tip 500 (which had been previously equilibrated using 10 mL buffer QBT) and allowed to flow under gravity pressure. Bound DNA was washed twice with 30 mL buffer QC then eluted with 15 mL buffer QF. 10.5 mL isopropanol was added to the eluted DNA and mixed well then centrifuged (17,000 x g, at 4 °C for 40 minutes). Precipitated DNA was washed once with 70 % ethanol, air-dried and resuspended in an appropriate volume of TE buffer.

2.2.13 Optical density measurement of DNA

The concentration of DNA was determined via measurement of the optical density at 260 nm, using a quartz cuvette. A Genova uv/vis spectrophotometer

(Jenway Ltd., Dunmow, Essex, U.K.) was used and an optical density reading of 1.0 was assumed to be equivalent to a concentration of 50 µg/mL.

2.2.14 DNA sequencing

DNA sequencing reactions were performed in 0.2 mL thin-walled tubes (Anachem Ltd.) for analysis using either 373A or 3100 automated DNA sequencer (Applied Biosystems, Warrington, Cheshire, U.K.)

For analysis using 373A sequencer, each reaction comprised 5 pmol sequencing primer, 1.0-1.5 µg plasmid DNA, 1.5 µL sequencing buffer and 1.5 µL Big Dye Terminator Reaction Ready sequencing premix (Applied Biosystems). Distilled water was added to bring the volume to 7.5 µL and the reaction was cycled under the following conditions:

96 °C	30 seconds] x 25 cycles
96 °C	10 seconds	
50 °C	5 seconds	
60 °C	4 minutes	
4 °C	constant	

The DNA was then precipitated by the addition of 2 µL 3 M sodium acetate (pH 4.6) and 50 µL 95 % ethanol. Samples were incubated on ice for 10 minutes and centrifuged at maximum speed (16,100 x g) for 15 minutes. The supernatant was removed and the pellet was air-dried, then resuspended in sample loading buffer.

For analysis using 3100 sequencer, reactions were prepared in the same manner as described above, except that 250 ng DNA was used per reaction. After cycling, samples were batch precipitated by the addition of 0.76 µL 3 M sodium acetate (pH 4.6) and 19.23 µL 95 % ethanol to each tube. Samples were

incubated at room temperature for 15 minutes and centrifuged at $1,500 \times g$ for 45 minutes at 4°C . The resultant pellets were washed with 70% ethanol, air dried and resuspended in $10 \mu\text{L}$ formamide.

2.2.15 Isolation of genomic DNA from mammalian cells

Genomic DNA was prepared from CHO-K1 cells using QIAGEN DNeasy kit, in accordance with the manufacturer's recommendations; centrifugation steps were performed using a benchtop centrifuge at maximum speed ($16,100 \times g$). In brief, approximately 5×10^6 cells were resuspended in $200 \mu\text{L}$ PBS then $20 \mu\text{L}$ proteinase K and $200 \mu\text{L}$ buffer AL added to the sample which was mixed by vortexing before incubating for 10 minutes at 70°C . $200 \mu\text{L}$ absolute ethanol was added and mixed by vortexing and the sample applied to a DNeasy spin column, which was placed in a collection tube and centrifuged for 1 minute. Bound DNA was washed first with $500 \mu\text{L}$ AW1 then with $500 \mu\text{L}$ AW2 buffers; before each wash step the spin column was placed into a fresh collection tube and centrifuged for 1 minute (AW1 wash) or 3 minutes (AW2 wash). DNA was eluted from the column by the application of $200 \mu\text{L}$ buffer AE and was collected by centrifugation after an incubation of 1 minute. The elution step was repeated and the eluates combined. Genomic DNA was stored at 4°C .

2.2.16 Isolation of total RNA from mammalian cells

Total RNA was prepared from CHO-K1 cells using QIAGEN RNeasy Mini kit, in accordance with the manufacturer's recommendations; centrifugation steps were performed using a benchtop centrifuge at maximum speed ($16,100 \times g$). Briefly, approximately 4×10^6 cells, which had been washed with PBS, were lysed in $350 \mu\text{L}$ buffer RLT (which had been supplemented with 143 mM

2-mercaptoethanol) and mixed by vortexing. The lysate was homogenised by application to a QIAshredder column, which was placed in a 2 mL collection tube and centrifuged for 2 minutes. 350 μ L 70 % ethanol was added to the homogenised lysate, mixed by pipetting and then applied to an RNeasy Mini column, which was placed in a 2 mL collection tube. The sample was bound to the column by centrifugation for 15 seconds and then washed with 700 μ L buffer RW1 followed by 2 x 500 μ L washes with buffer RPE; centrifugation followed each wash step. Bound RNA was eluted by the application of 50 μ L RNase-free water and collected by centrifugation for 1 minute; the elution was repeated and the eluates were pooled.

Purified total RNA was treated with amplification grade deoxyribonuclease I (DNase I) to eliminate any contaminating genomic DNA. Approximately 5 μ g RNA was treated in a 50 μ L reaction, which also contained 5 μ L 10x DNase I reaction buffer, 5 units DNase I and an appropriate volume of DEPC-treated water. After incubation at room temperature for 15 minutes, the reaction was terminated by the addition of EDTA to a final concentration of 2.2 mM, followed by heat inactivation of the enzyme at 65 °C for 10 minutes.

2.2.17 Synthesis of first strand cDNA by RT-PCR

First strand cDNA was prepared from CHO-K1 total RNA (preparation of which is described in this chapter, section 2.16) using Advantage RT-for-PCR kit, following the manufacturer's protocol. In essence, approximately 1 μ g total RNA was mixed with oligo dT primer and heated for 2 minutes at 70 °C then immediately cooled on ice. 5x reaction buffer, dNTPs, recombinant RNase inhibitor and MMLV reverse transcriptase were added to give a 20 μ L reaction

which was incubated at 42 °C for 1 hour then heated at 94 °C to terminate the reaction, denature RNA-cDNA hybrids and destroy DNase activity. The resultant cDNA was stored at -80 °C until use and 2 µL used as template in 20 µL gene specific amplifications, which were carried out as described in this chapter, section 2.1.

2.3 Cell culture and cell biology methods

2.3.1 General cell culture

HEK-293 and CHO-K1 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 2 mM L-glutamine, 10 % (v/v) foetal calf serum and 1x non essential amino acids ("complete DMEM"). The CHO-EcR cell line was maintained in Ham's F-12 Nutrient Mix, supplemented with 10 % (v/v) foetal calf serum, 2 mM L-glutamine and 250 µg/mL Zeocin ("Complete F12"). Stable cell lines were cultured in the appropriate medium, to which 7.5 µg/mL Puromycin (CHO-K1) or 500 µg/mL Geneticin (CHO-EcR) had been added.

CHO and HEK cells adhere to tissue culture plastics and were normally cultured in T175 flasks. Routine passage of cells was performed using trypsin-EDTA to dislodge the adherent cell monolayer; medium was removed from the flask and the adherent cells were washed once with 10 mL Dulbecco's PBS before 3 mL trypsin-EDTA in Hank's Balanced Salt Solution was added. Cells were incubated at 37 °C for approximately 5 minutes until cells could be dislodged by tapping the flask. 7 mL "complete DMEM" was added and an appropriate volume of cell suspension transferred to a fresh flask together with 40 mL medium which was incubated at 37 °C with 5 % CO₂. Cells were subcultured at

a ratio which achieved confluence after approximately 4 days: CHO-K1 cells were split 1 in 100; CHO-EcR were split 1 in 50; HEK-293 were split 1 in 10.

Cell lines were cryopreserved for long term storage. Cell monolayers were removed by trypsinisation, centrifuged, the cell pellet was resuspended in freezing medium (90 % (v/v) foetal calf serum + 10 % (v/v) DMSO) and dispensed into cryovials. Aliquots of cells were packed in expanded polystyrene and chilled to -70 °C before transfer of the aliquots to liquid nitrogen.

2.3.2 Transient transfection of HEK-293 cells

HEK-293 cells were transiently transfected to express $\alpha_{2A}AR-G\alpha_{i1}$ fusion protein, which was to be used as a positive control in the [^{35}S]GTP γ S binding assay. Cells were seeded into poly-D-lysine coated T175 flasks (2×10^7 cells/flask) on the day prior to transfection. The resulting sub-confluent cells were transfected using Lipofectamine reagent (Invitrogen Ltd.) in accordance with the manufacturer's protocol. Briefly, 36 μ g plasmid DNA was mixed with 1.8 mL Opti-Mem Reduced Serum Medium to yield Solution A; 144 μ L Lipofectamine was mixed with 1.8 mL Opti-Mem to produce solution B. Solutions A and B were combined and incubated at room temperature for 30 minutes to allow DNA-liposome complexes to form. 14.4 mL Opti-Mem was added to the complexes and mixed gently. Diluted complexes were overlaid onto the cells (which had been washed once with Opti-Mem) and incubated for 6 hours at 37 °C with 5 % CO₂ after which time they were removed and 50 mL fresh "complete DMEM" added to the cells. Cells were grown for 2-3 days at 37 °C with 5 % CO₂ before being harvested.

2.3.3 Generation of stable cell lines

CHO-K1 cells were stably transfected to express HA-S1P₄, HA-S1P₄-Gα_{i1}(C³⁵¹I) or HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) proteins. Cells were transfected essentially as described in this chapter, section 3.2 with the following modifications: 0.67 x 10⁶ cells were seeded into a T25 flask on the day prior to transfection, such that they were 50-80 % confluent on the day of transfection. Solution A was prepared by mixing 5.3 µg plasmid DNA with 267 µL Opti-Mem Reduced Serum Medium; 21.3 µL Lipofectamine reagent was mixed with 267 µL Opti-Mem to yield Solution B. After the combination of solutions A and B and incubation to allow formation of complexes, 6.4 mL Opti-Mem was added and the diluted complexes laid over cells which had been washed once with Opti-Mem. Cells were incubated with the complexes for 6 hours at 37 °C with 5 % CO₂ then the complexes were removed and replaced with 5 mL “complete DMEM”. 2-3 days post transfection, cells were detached using trypsin, counted, resuspended in “complete DMEM” supplemented with 7.5 µg/mL Puromycin and seeded into a number of 96-well plates (200 µL/well); seeding densities of 10, 100 and 1000 cells/well were used. The plates were incubated at 37 °C with 5 % CO₂ for 2-3 weeks before inspection of wells to identify those containing a single colony of Puromycin-resistant cells. Such colonies were detached into 200 µL trypsin and expanded into 24-well plates (1 mL/well); when confluent, cells were seeded into 2 copies of a 6-well plate (one copy was then used to test for expression of the recombinant protein of interest). Positive clones (identified using FACS analysis, described in this chapter, section 3.5) were then expanded into T175 flasks (via T25 flasks) and frozen stocks prepared. A mock transfection was set up in parallel to each transfection; this was to ensure that Puromycin-resistant

colonies were the result of transfection with plasmid containing Puromycin-resistance gene.

The CHO-EcR cell line was stably transfected to express HA-S1P₄ using Lipofectamine 2000 (Invitrogen Ltd.) as the transfection reagent and following the manufacturer's protocol. Essentially, CHO-EcR cells were seeded in T75 flasks such that they were 90-95 % confluent on the day of transfection. 114 µL Lipofectamine 2000 was mixed with 1.9 mL Opti-Mem Reduced Serum Medium and incubated for 5 minutes at room temperature; this was Solution A. 38 µg plasmid DNA was mixed with 1.9 mL Opti-Mem to form Solution B. Solutions A and B were mixed and incubated at room temperature for 20 minutes to allow formation of DNA-liposome complexes. Cells were washed once with Opti-Mem medium and 8 mL fresh Opti-Mem was added to the cells before the complexes were laid onto cells. The cells were incubated with complexes for 6 hours at 37 °C with 5 % CO₂ after which time complexes were removed and 15 mL "complete F12" medium added to each flask. 2-3 days post-transfection, cells were trypsinised, counted and seeded into 96 well plates in "complete F12" medium supplemented with 500 µg/mL Geneticin and single colonies isolated and expanded as described previously in this chapter. A mock transfection was set up in parallel.

2.3.4 Induction of HA-S1P₄ expression in ecdysone-inducible CHO-EcR cell line

Small scale experiments used to profile this expression system were set up in 24-well tissue culture plates using CHO-EcR cells which had been stably transfected to express HA-S1P₄ under the control of the ecdysone receptor. Cells were normally seeded on the day prior to an experiment with 1.6×10^5 cells per well,

in “complete F12” medium. Plates were placed on an orbital shaker for 30 minutes to aid even settling of the cells and then transferred to 37 °C with 5 % CO₂ for 3 hours to allow attachment to the tissue culture plastic. Medium was then removed and the attached cells washed once with PBS before serum-free F12 medium containing the appropriate concentration of induction agent was applied to the cells. The induction agents, ponasterone A and muristerone A, were prepared as 10 mM DMSO stocks. Cells were then assayed to determine cell-surface receptor expression (this chapter, section 3.5) 24 hours after application of the induction agent. For expression timecourse experiments, cells were seeded in 24 well plates at 0.6×10^5 cells per well three days prior to assay and medium replaced with serum-free F12 medium containing induction agent at appropriate timepoints. Cells were assayed to determine cell-surface receptor expression as normal.

2.3.5 Detection of cell-surface proteins (FACS analysis)

Cells ($>1 \times 10^5$) were detached from tissue culture plastics using cell dissociation solution (Sigma) and mixed with FACS buffer (PBS containing 3 % (v/v) FCS, 0.1 % (w/v) NaN₃) in 5 mL polypropylene Falcon tubes (BD Biosciences, Cowley, Oxon., U.K.). After collection by centrifugation (4 minutes at 400 x g), cells were resuspended in 100 µL FACS buffer in the presence of approximately 0.5 µg/mL rat anti-HA-Fluorescein antibody or an isotype matched control antibody, and incubated for 1 hour at 4 °C, in the dark. Cells were washed with 3 mL chilled FACS buffer and resuspended in 200 µL of the same. Staining data was measured using FACScan or FACScalibur flow cytometers (BD

Biosciences); staining with the specific antibody was compared to that obtained with the isotype matched control antibody.

2.3.6 Fluorescence microscopy

Cells were non-enzymatically detached from tissue culture plastics using cell dissociation solution and seeded into 8 chamber tissue culture treated glass slides (2×10^4 cells/chamber) in the appropriate complete medium. Where appropriate, the induction agent ponasterone A was added to medium. 24 hours after seeding, cells were washed well with medium and stained in complete medium containing the rat anti-HA-Fluorescein antibody (0.5 $\mu\text{g/mL}$) for 30 minutes in the dark. The nuclear stain DAPI (4',6'-diamidino-2-phenylindole hydrochloride) was added to the medium for the final 10 minutes (10 $\mu\text{g/mL}$). Cells were washed with medium and the slides mounted using ProLong Antifade Kit (Molecular Probes, Leiden, Holland). Fluorescent images were observed using a Leica DMRXA fluorescence microscope with oil immersion lenses. Fluorescein was excited at 495 nm (520 nm emission) and excitation of the DAPI stain was at 420 nm (345 nm emission). Images were manipulated using Openlab 2.2.0 deconvolution software (Improvision, Coventry, U. K.).

2.4 Protein methods

2.4.1 Preparation of cell membranes

CHO cell lines for use in S1P-receptor activation assays were grown in T175 flasks or 2 L roller bottles and transferred to serum-free medium approximately 24 hours prior to harvest. Briefly, medium was aspirated and cells washed twice with PBS before replacement with serum-free DMEM (containing 100 $\mu\text{g/mL}$ pertussis toxin, where appropriate). HEK-293 cells, which had been transiently

transfected to express $\alpha_{2A}AR-G\alpha_{i1}(C^{351}I)$ fusion protein, were grown in “complete” DMEM. To harvest cells, growth medium was removed and the adherent cells washed with PBS. Cells were dislodged from culture vessels using cell dissociation solution and collected by centrifugation; the resultant cell pellet was washed with PBS. Cells were resuspended in an appropriate volume of membrane preparation buffer (20 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, “complete” protease inhibitors (Roche Molecular Biochemicals)). For small scale membrane preparations, cells were ruptured by 35 strokes using a hand held Wheaton tight fit glass douncer; larger scale preparations were homogenised in a nitrogen cavitation chamber (500 psi for 20 minutes). Unbroken cells and nuclei were removed by centrifugation (500 x g, 10 minutes at 4 °C) and the supernatant fraction was centrifuged at 45,000 x g for 45 minutes at 4 °C. Membrane pellets were resuspended in membrane preparation buffer, titrated through a fine gauge needle, aliquotted and stored at -80 °C until required.

2.4.2 Determination of protein concentration

The protein concentration of membrane preparations was determined using Micro BCA Protein Assay Reagents (Perbio Science U.K. Ltd., Tattenhall, Cheshire, U.K.) in accordance with the manufacturer’s protocol. Membrane preparation buffer (this chapter, section 5.2) was used as the diluent for BSA standards and samples.

2.4.3 Immunological detection of protein (Western blotting)

Samples (typically membrane preparations) were prepared for Western blot analysis by the addition of an equal volume of 2x protein sample buffer and

incubation for 1 hour at room temperature. Approximately 10 µg each sample was loaded onto 4-20 % Tris-Glycine pre-cast polyacrylamide gel alongside prestained molecular weight markers. Gels were electrophoresed at 100 V / 35 mA for approximately 90 minutes, until the dye front had reached the bottom of the gel. Proteins were transferred to Immobilon-P PVDF membrane, which had been pre-wetted in methanol, overnight at 80 mA; the use of prestained molecular weight markers allowed judgement of the success of protein transfer. The resultant blot was blocked in PBS containing 2.5% (w/v) Marvel for 1 hour at room temperature, with constant agitation. Antibodies were prepared in PBS containing 1 % (w/v) Marvel and 0.1 % (v/v) Tween-20. The membrane was incubated with the primary antibody for 4 hours at room temperature, then washed three times in PBS containing 0.1 % (v/v) Tween-20 (5 minutes per wash) before incubation with the appropriate horseradish peroxidase-conjugated secondary antibody for approximately 2 hours at room temperature. After a further three washes (10 minutes per wash), the blot was developed using Pierce SuperSignal reagents (Perbio Science), in accordance with the manufacturer's instructions. The signal was detected using x-ray film, which was processed using an X-OMat automatic developer (Kodak Ltd., Hemel Hempstead, Herts., U. K.).

2.5 Receptor activity assays

2.5.1 Handling of receptor agonists

Lysophospholipid solutions were handled using siliconised Eppendorfs and pipette tips to minimise losses due to adhesion to plastics. Sphingosine-1-phosphate was dissolved in methanol to give a 1 µM solution, this required heating at 60 °C and

repeated vortexing and sonication to disperse the solid. 100 μ L aliquots (equivalent to 0.1 μ moles compound) were dispensed into 2 mL Wheaton brown glass vials (Sigma) and the solvent gently evaporated under a stream of nitrogen. Aliquots were then stored at -80 °C prior to use. To reconstitute an aliquot of S1P, 250 μ L “assay buffer” (this chapter, section 5.2) supplemented with 1 % (w/v) fatty acid free BSA was added and incubated at 37 °C for 30 minutes, with repeated vortexing. A further 1 in 4 dilution in assay buffer supplemented with 1 % (w/v) fatty acid free BSA yielded a 100 μ M solution of S1P for use in receptor activation assays.

Lysophosphatidic acid (18:1) was prepared as a 2 mM DMSO solution (repeated vortexing and sonication were used to disperse the solid) and aliquotted for storage at -20 °C. 16:0 and 14:0 lysophosphatidic acids were prepared in 1:1 ethanol:water as 2 mM stock solutions, aliquotted and stored at -20 °C until use. Dilution of these stock solutions with “assay buffer” (this chapter, section 5.2) supplemented with 1 % (w/v) fatty acid free BSA gave a solution of LPA, which could be used in assays.

The α_{2A} AR agonist, UK14304 was prepared as a 5 mM stock solution in 1:1 DMSO:water and aliquots stored at -80 °C.

Compounds FTY720 and AAL were synthesised by the medicinal chemistry department of Celltech R+D using synthetic schemes described previously [Kiuchi *et al.*, 2000]. Each compound was then prepared as 1mM stock solution in ethanol and stored at -20 °C. *In vitro* phosphorylation of FTY720 and AAL was achieved via incubation with rabbit blood (essentially as described by

Mandala *et al.*, [2002]). Heparinised venous rabbit blood was mixed with three volumes of RPMI medium (supplemented with 10 % FCS, 2 mM L-glutamine and 1x non essential amino acids). FTY720 or AAL compounds were added to the diluted blood and the phosphorylation reaction incubated for the stated time at 37 °C, without additional CO₂. Post reaction, four volumes of acetonitrile were added and mixed well before centrifugation at approximately 2000 x g for 15 minutes. The supernatant was then analysed by liquid chromatography-mass spectrometry (LC-MS).

2.5.2 [³⁵S]GTPγS binding assay

[³⁵S]GTPγS binding experiments were performed in accordance with the method described by Stevens *et al.*, [2001]. Reagents were prepared in “assay buffer” (20 mM HEPES (pH 7.4), 3 mM MgCl₂, 100 mM NaCl). Membranes (20 µg/point) were incubated with or without ligand for an appropriate time at 30 °C in “assay buffer” containing [³⁵S]GTPγS (100 nCi/point), and an appropriate concentration of GDP, in a final volume of 100 µL/point. Where indicated, saponin (20 µg/point) was included. Lysophospholipid ligands were prepared in “assay buffer” supplemented with 1 % (w/v) fatty acid free BSA (this chapter, section 5.2), such that the final BSA concentration in the assay was 0.1 % (w/v). UK14304, an α_{2A}AR agonist, was diluted in “assay buffer” and used at a final concentration of 1 µM.

Following incubation, 500 µL chilled “assay buffer” containing “complete” protease inhibitors was added to each tube and membranes collected by centrifugation (14,000 rpm (16,100 x g) for 15 minutes at 4 °C). Membranes were solubilised for 1 hour at 4 °C, mixing end-over-end, in 50 µL 0.4 % (w/v)

SDS in chilled “solubilisation buffer” (100 mM Tris-HCl (pH 7.4 at 4 °C), 200 mM NaCl, 1 mM EDTA, “complete” protease inhibitors and 1.25 % (v/v) NP-40). This solution was then precleared by the addition of 60 µL/point of a mixture containing normal rabbit serum (diluted in “solubilisation buffer” to give 1 in 100 final concentration) and protein G Sepharose beads (6 µL/point diluted in “bead buffer” (2 % (w/v) BSA, containing 0.1 % (w/v) NaN₃ and “complete” protease inhibitors)) and mixed for 1 hour at 4 °C. Beads were pelleted by centrifugation (14,000 rpm for 5 minutes at 4 °C) and the supernatant was immunoprecipitated with SG1 antiserum, which directed against G $\alpha_{i1/2}$ G proteins. 50 µL of a mix containing SG1 (diluted to give 1 in 200 final concentration in “solubilisation buffer”) and protein G Sepharose beads (5 µL/point diluted in “bead buffer”) was added to each tube and mixed overnight at 4 °C. Beads were collected by centrifugation and washed twice with 500 µL “solubilisation buffer”. 1 mL Ultima Gold XR scintillation fluid (Perkin Elmer, Cambridge, U.K.) was added to each tube and bound radioactivity measured using liquid scintillation spectrometry. Non-specific binding in the presence of 100 µM GTP γ S was also determined in each experiment.

2.5.3 Data analysis

Analysis of numerical data was performed using Graphpad Prism, version 3.00. Data were corrected for non-specific binding and where appropriate, fitted using nonlinear (sigmoidal dose-response, fixed slope) regression. This method is reliant on the presence of at least two data points on the top and bottom sections of the curve, and at least one data point on the sigmoidal section of the curve in order to generate a dose-response curve; where this does not occur, interpretation

of the resultant EC₅₀ value should be cautious. Results were expressed as the mean of triplicate determinations \pm SEM. Typically, assays were performed at least three times, using membranes prepared from at least two individual cell passages. Statistical tests applied to data were one-way ANOVA, with Dunnett's multiple comparison post test. Investigations of the statistical relationship between two data sets were performed using Pearson's correlation.

Chapter 3 – Cloning of Human S1P₄ and Production of Expression Constructs

3.1 Introduction

This chapter describes the cloning and construction of plasmids for expression in mammalian cells and the application of RT-PCR to determine the profile of endogenous S1P and LPA receptor mRNA expression in CHO-K1 cells.

The S1P₄ receptor was cloned from a human PBMC cDNA library and modified to contain an amino-terminal HA epitope-tag. It was also constructed as an in-frame fusion between HA-tagged S1P₄ and a pertussis toxin resistant G α_{i1} (C³⁵¹I) G protein. These constructs were made using the pIRESpuro mammalian expression vector (Figure 3.1). HA-S1P₄ and the HA-S1P₄-G α_{i1} (C³⁵¹I) fusion protein were stably expressed in CHO-K1 cells and used in functional assays as described in chapters 4 and 6.

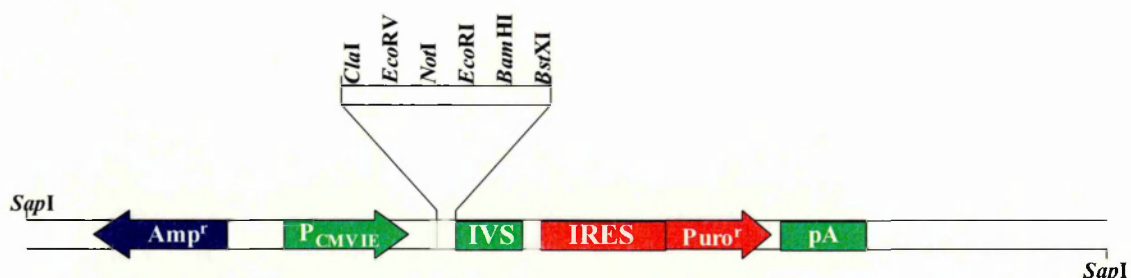


Figure 3. 1

Schematic diagram of the pIRESpuro mammalian expression vector.

The vector is approximately 5.2 kb in size and includes the internal ribosome entry site (IRES), which permits the translation of two open reading frames from one mRNA. This expression cassette contains the human cytomegalovirus (CMV) major intermediate early promoter/enhancer which drives expression of cloned genes; a multiple cloning site for insertion of cloned genes; a synthetic intervening sequence (IVS) intron to enhance mRNA stability; the encephalomyocarditis virus (ECMV) IRES followed by the Puromycin resistance marker; bovine growth hormone polyadenylation signal for proper processing of the 3' end of the cloned mRNA. The vector backbone also contains the β -lactamase gene to confer resistance to ampicillin and the pBR322 origin of replication for propagation in *E. Coli*.

HA-tagged S1P₄ was also constructed in the pIND expression vector (Figure 3.2) and stably transfected into CHO-EcR cells for the inducible expression studies described in chapter 5.

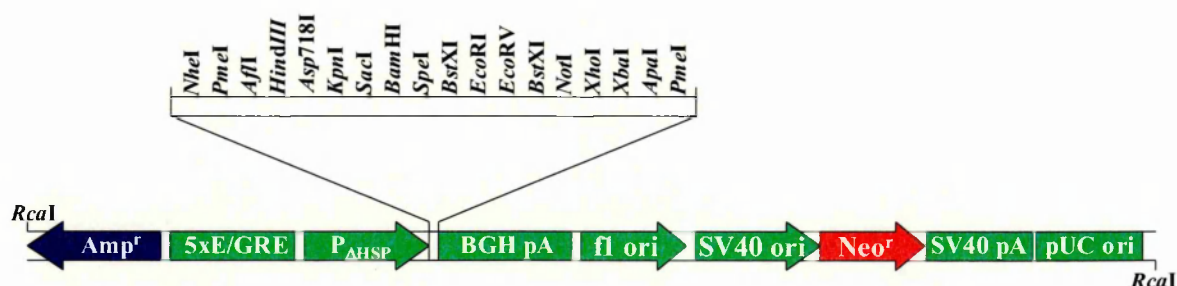


Figure 3. 2

Schematic diagram of the pIND ecdysone-inducible mammalian expression vector.

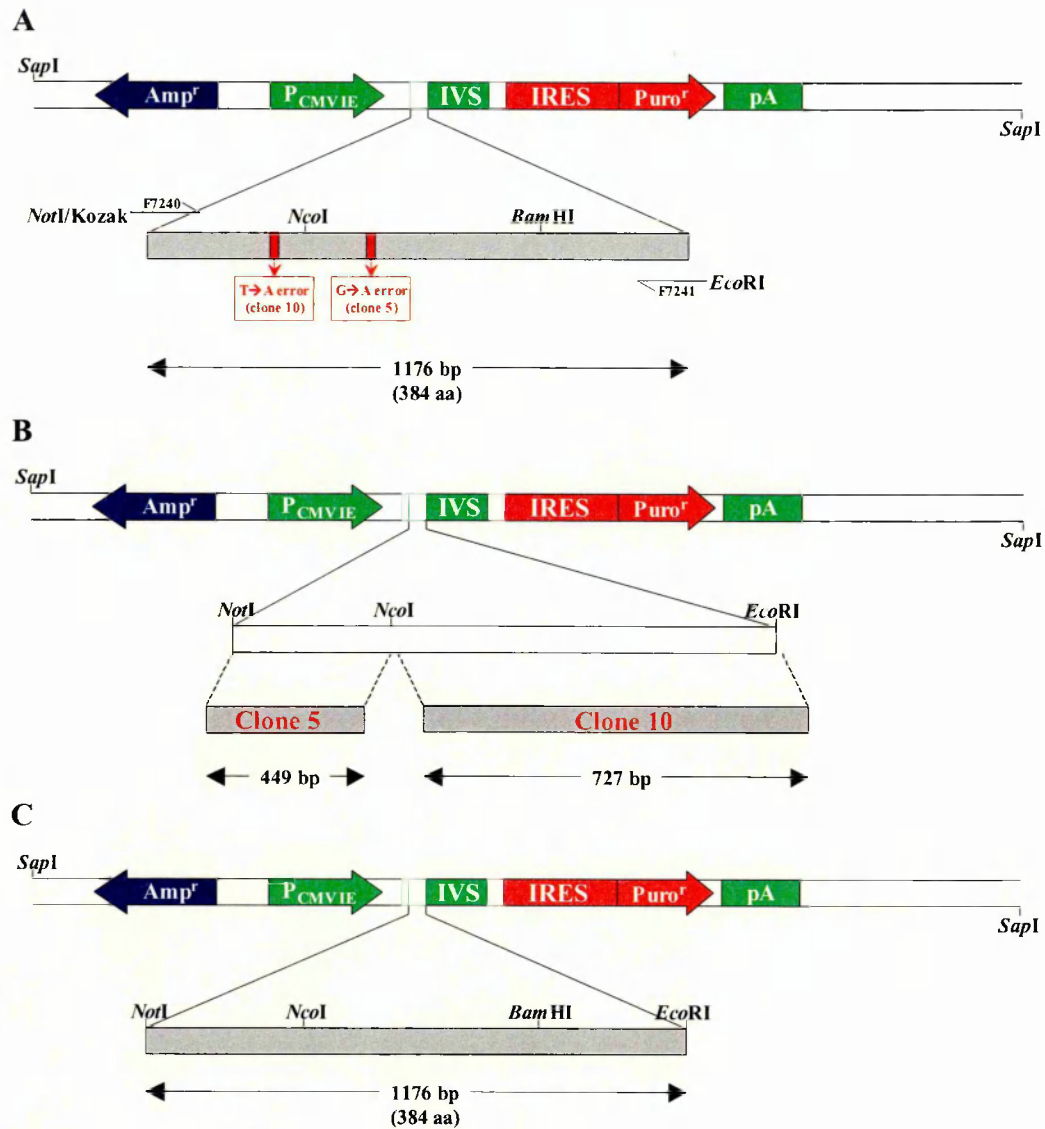
This vector is approximately 5.0 kb in size and contains five ecdysone/glucocorticoid receptor elements (E/GRE), which allow binding of the modified ecdysone-receptor for activation of gene transcription and inducible expression of the gene of interest. Its other essential elements include the minimal heat shock promoter, which drives expression of the gene of interest; a multiple cloning site; bovine growth hormone (BGH) polyadenylation signal to allow efficient transcription termination and polyadenylation of cloned mRNA; f1 origin of replication; SV40 early promoter and origin of replication to drive high level expression of the neomycin resistance gene, which confers resistance to the antibiotic Geneticin and SV40 polyadenylation signal. The vector also contains β -lactamase gene and pUC origin for high copy number replication and propagation in *E. Coli*.

Residue ¹²² (glutamic acid) of S1P₄ was mutated to glutamine to allow investigation into the role of this residue in the ligand preference of S1P₄. This mutant was constructed with an HA-epitope tag as an in-frame fusion with the pertussis toxin resistant mutant of G α_{i1} (C³⁵¹I) G protein, using the pIRESpuro expression vector. These proteins were then stably expressed in CHO-K1 cells and their response in functional assays measured as described in chapter 6.

3.2 Results

3.2.1 Cloning of HA-tagged S1P₄

In order to clone S1P₄, the published sequence (Genbank accession number AJ000479) was used to design primers F7240 and F7241 to allow amplification of the coding sequence of the S1P₄ gene. The 5' oligonucleotide, F7240, contained a *NotI* restriction site to facilitate cloning and a partial Kozak translational initiation sequence for optimal gene expression, upstream of the initiator methionine. F7241, the 3' oligonucleotide, contained the terminal coding sequence of S1P₄, an additional stop codon and an *EcoRI* restriction site. S1P₄ was amplified from a human peripheral blood mononuclear cell (PBMC) cDNA library using PCR. Since the S1P₄ sequence was GC rich (approximately 67 % GC content), it was necessary to use a "GC-rich" PCR kit to amplify this sequence. The resultant PCR product was digested with the appropriate restriction enzymes to yield a fragment of approximately 1152 bp. This fragment was gel-purified and ligated into the mammalian expression vector, pIRESpuro (Figure 3.3 (A)), which had been digested with *NotI* and *EcoRI*, then transformed into chemically competent *E. Coli*. Single colonies were cultured and plasmid DNA purified and the presence of insert verified by restriction digest and analysis by gel electrophoresis. The entire insert was sequenced using a combination of sequencing primers designed from the published S1P₄ sequence, and vector-specific primers. A number of clones were sequenced and were all found to contain PCR errors, which may have been due to the use of modifiers in the PCR mix and were necessary for amplification of this GC rich sequence. These were identified as PCR errors since they were random errors

**Figure 3. 3****Construction of S1P₄/pIRES.**

S1P₄ was amplified from a human peripheral blood mononuclear cell (PBMC) cDNA library using the 5' oligonucleotide F7240, which contained the *NotI* restriction site and a partial Kozak sequence, and the 3' oligonucleotide F7241, which contained an *EcoRI* restriction site. The fragment was digested with *NotI* and *EcoRI* and ligated into pIRES (A). Due to the presence of PCR errors in the clones which were sequenced, it was necessary to generate an authentic construct via restriction digest and three-way ligation of two clones, each containing a single random PCR error either side of the unique *NcoI* site located at position 437, into the pIRES vector (C). Two unique restriction sites located within the S1P₄ gene: *NcoI* and *BamHI*, are also shown. These sites were used to facilitate construction of this construct, epitope-tagged S1P₄, a fusion of S1P₄ with the Gα_{i1} G protein and a fusion of S1P₄(E¹²²Q) with the pertussis toxin-insensitive Gα_{i1}(C³⁵¹I) G protein.

that were not present in every clone sequenced. Two clones were identified which each contained a single error: clone number 10 had an error at position 348, whilst in clone number 5 there was a single error at position 642. A unique *NcoI* restriction site at position 437 was utilised to produce a complete error free construct. DNA from each clone was digested with appropriate restriction enzymes (clone 5 with *NotI/NcoI*; clone 10 with *NcoI/EcoRI*) to excise the error-free section of the amplified sequence (Figure 3.3 (B)). These 5' and 3' portions were then ligated into pIRESpuro, which had been digested with *NotI* and *EcoRI* enzymes. DNA sequencing was used to confirm the absence of errors in the resultant construct and the sequence obtained was identical to that described by Graeler *et al.*, [1998] and this plasmid was named pIRES/S1P₄, (Figure 3.3 (C)).

It was necessary to epitope tag the S1P₄ sequence since no antibodies to this receptor were available and, being an orphan GPCR at the time this work was undertaken, there was no possibility of detecting expression of protein using a functional assay. Therefore, pIRES/S1P₄ was modified by PCR. The 5' oligonucleotide F12582 contained a *NotI* restriction site for cloning, a partial Kozak sequence and the HA-epitope (YPYDVPDYA) inserted between the first and second codons of the S1P₄ coding sequence. The 3' oligonucleotide, F12581, was designed across the unique *NcoI* site present in the S1P₄ sequence such that only the 5' end of the gene would be amplified (Figure 3.4 (A)). The product of this PCR was digested with *NotI* and *NcoI* restriction enzymes to yield a 449 bp fragment. The 3' end of the S1P₄ coding sequence with which this 5' fragment was to be ligated was produced by digestion of the pIRES/ S1P₄ plasmid with *NcoI* and *EcoRI* restriction enzymes and gel purification (Figure

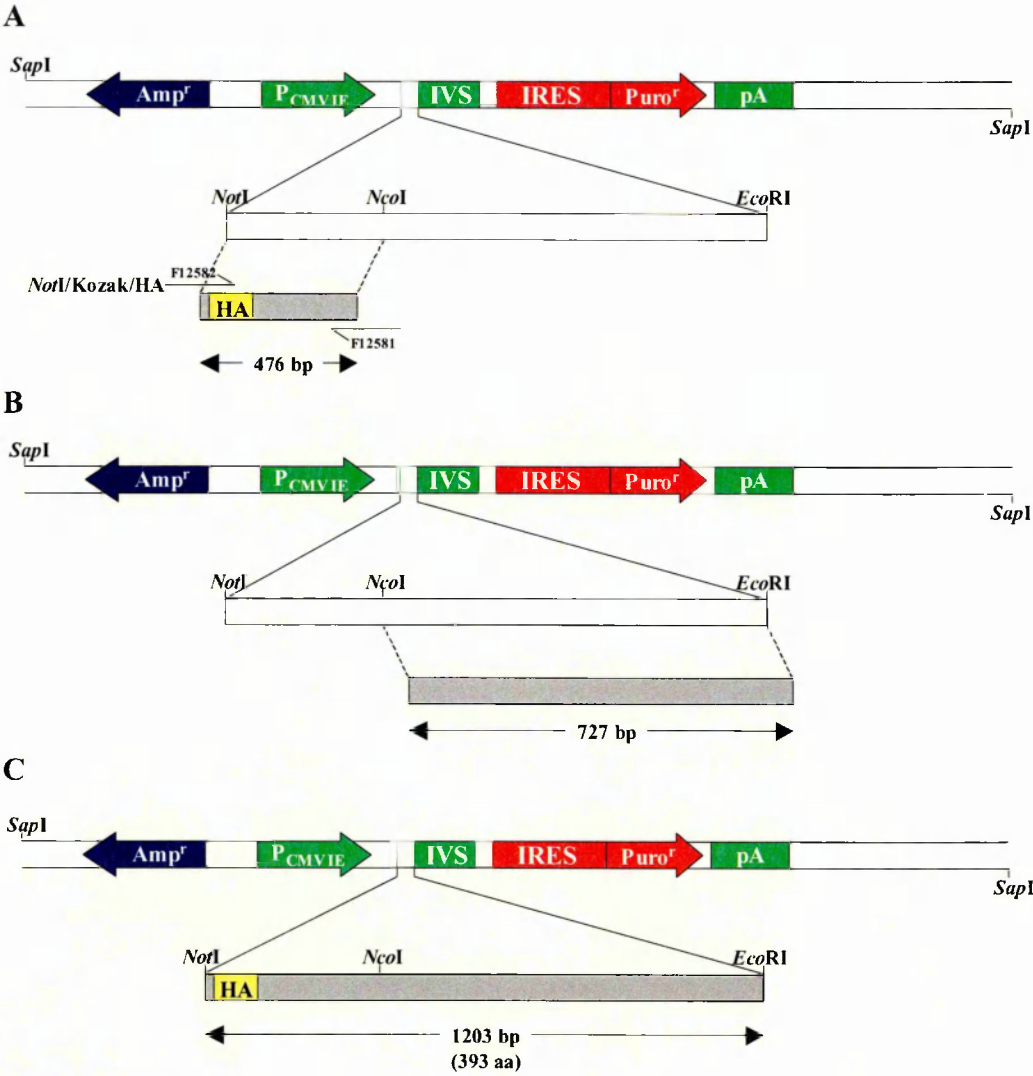


Figure 3. 4
Construction of HA-S1P₄/pIRES.

S1P₄ was HA-epitope tagged by PCR amplification of the 5' portion of the sequence, using the forward oligonucleotide F12582, which contained the HA epitope sequence (YPYDVDPDYA) immediately after the initiator methionine, as well as a partial Kozak sequence and *NotI* restriction site, and the reverse oligonucleotide F12581 which utilised the unique *NcoI* site present in the S1P₄ sequence. This fragment (A) was joined with fragment (B), produced by *NcoI/EcoRI* restriction digest of S1P₄/pIRES, in a three-way ligation reaction with *NotI/EcoRI* digested pIRES to yield HA-S1P₄/pIRES (C).

3.4 (B)). The complete construct was then generated by three-way ligation reaction of the PCR-generated HA-tagged 5' fragment, the restriction digest-produced 3' fragment and *NotI/EcoRI*-digested pIRES. Plasmid DNA was extracted from transformed *E. Coli* colonies and digested with restriction

enzymes to check for the presence of insert. The entire insert was sequenced with vector- and insert-specific primers and was found to be error free. This construct was named HA-S1P₄/pIRES (Figure 3.4 (C)) .

3.2.2 Generation of HA-tagged S1P₄-G α_{i1} (C³⁵¹I) fusion construct

The construct to express HA-S1P₄ fused to G α_{i1} , where the G protein contained the C³⁵¹I mutation and was therefore resistant to ADP-ribosylation by pertussis toxin, was produced in several stages. Initially, PCR was used to remove the stop codon present at the 3' end of the HA-S1P₄ coding sequence in the previously produced HA-S1P₄/pIRES construct. The forward oligonucleotide, F27384, which encompassed a unique *Bam*HI restriction site within the S1P₄ sequence, was used in conjunction with the reverse oligonucleotide, F36663, which allowed mutation of the stop codon to alanine and also contained the *Eco*RI restriction site. The resultant PCR product was digested with *Bam*HI and *Eco*RI restriction enzymes and gel purified to yield a fragment of approximately 290 bp (Figure 3.5 (A)). Digestion of HA-S1P₄/pIRES with *Not*I and *Bam*HI yielded the 910 bp 5' fragment of HA-S1P₄ (Figure 3.5 (B)) which was gel purified before three-way ligation with the PCR-generated 3' fragment and pIRES which had been cut with *Not*I and *Eco*RI. This ligation reaction was transformed into chemically competent *E. Coli* and plasmid DNA extracted from cultures of single colonies was analysed by restriction digest and gel electrophoresis to check for the presence of insert. The entire insert of a single clone was sequenced on both strands and found to be error free. This intermediate construct was named HA-S1P₄(No Stop)/pIRES and is shown in Figure 3.5 (C).

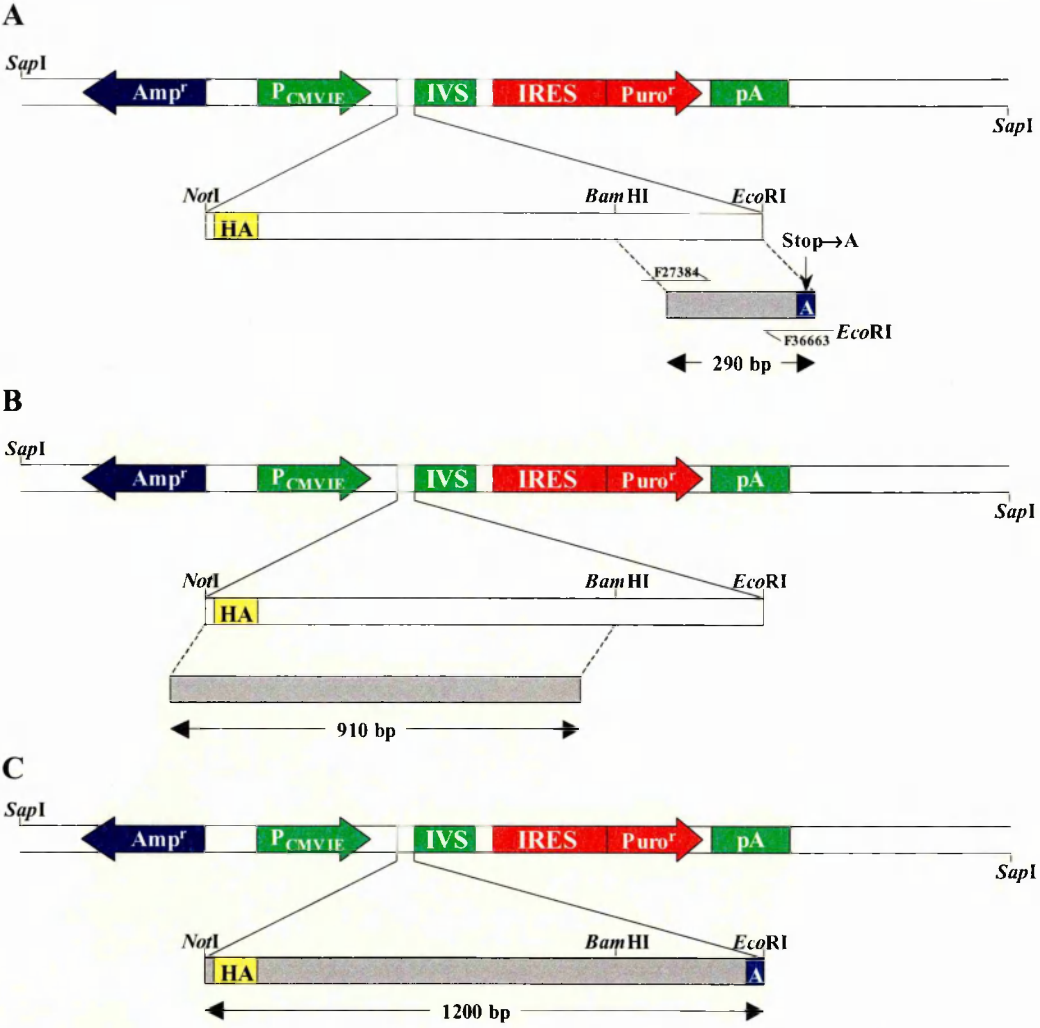


Figure 3.5
Construction of HA-S1P₄(No Stop)/pIRES.

A construct of HA-S1P₄ which lacked the 3' stop codon was generated by PCR amplification of a 3' fragment of HA-S1P₄ using the forward primer F27384, which encompassed a unique *Bam*HI site within the S1P₄ sequence, and the reverse oligonucleotide F36663, which mutated the stop codon to alanine and contained an *Eco*RI restriction site to facilitate cloning. Restriction digest and gel purification of the PCR product yielded a fragment of approximately 290 bp (A). This was ligated in a three-way reaction with fragment (B), which had been produced by *Not*I/*Bam*HI digestion of HA-S1P₄, and *Not*I/*Eco*RI-digested pIRES, to produce HA-S1P₄(No Stop)/pIRES (C).

The G α_{i1} (C³⁵¹I) G protein was modified by PCR such that it contained restriction sites which would allow in-frame fusion with the HA-S1P₄(No Stop) sequence. The forward oligonucleotide F36661 introduced an *Eco*RI restriction site before the initiator methionine, and the reverse oligonucleotide F36662 created a

*Bam*HI site after the stop codon of $G\alpha_{i1}(C^{351}I)$. Digestion of the PCR product with appropriate restriction enzymes and subsequent gel-purification, yielded a fragment of approximately 1083 bp which was ligated into pIRES, which itself had been digested with *Eco*RI and *Bam*HI. Chemically competent *E. Coli* were transformed with this ligation reaction and plasmid DNA extracted from cultures grown from single colonies. Analysis of this DNA by restriction digest and gel electrophoresis confirmed the presence of the insert, after which the insert of a single clone was sequenced on both strands, using $G\alpha_{i1}$ - and vector-specific sequencing primers. This clone was found to be error free and was named $G\alpha_{i1}(C^{351}I)/pIRES$ (shown in Figure 3.6).

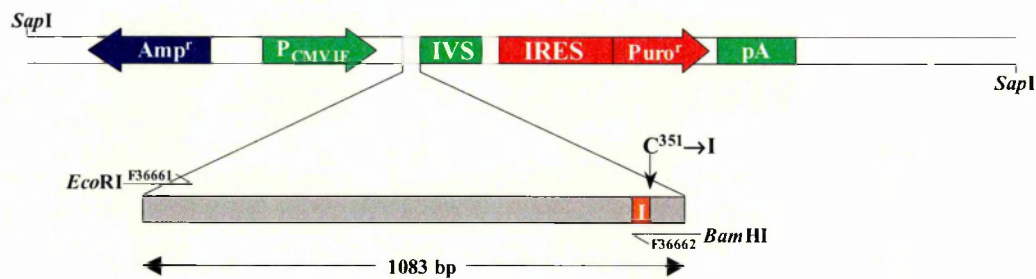


Figure 3. 6

Construction of $G\alpha_{i1}(C^{351}I)/pIRES$.

$G\alpha_{i1}(C^{351}I)$ was modified using PCR such that it contained an *Eco*RI restriction site at the 5' end and a *Bam*HI restriction site at the 3' end. These sites were present in the forward oligonucleotide, F36661, and the reverse oligonucleotide, F36662, respectively. Digestion of the PCR product with *Eco*RI and *Bam*HI yielded a fragment of approximately 1083 bp, which was ligated into pIRES, which had also been cut with *Eco*RI and *Bam*HI. This produced the $G\alpha_{i1}(C^{351}I)/pIRES$ construct.

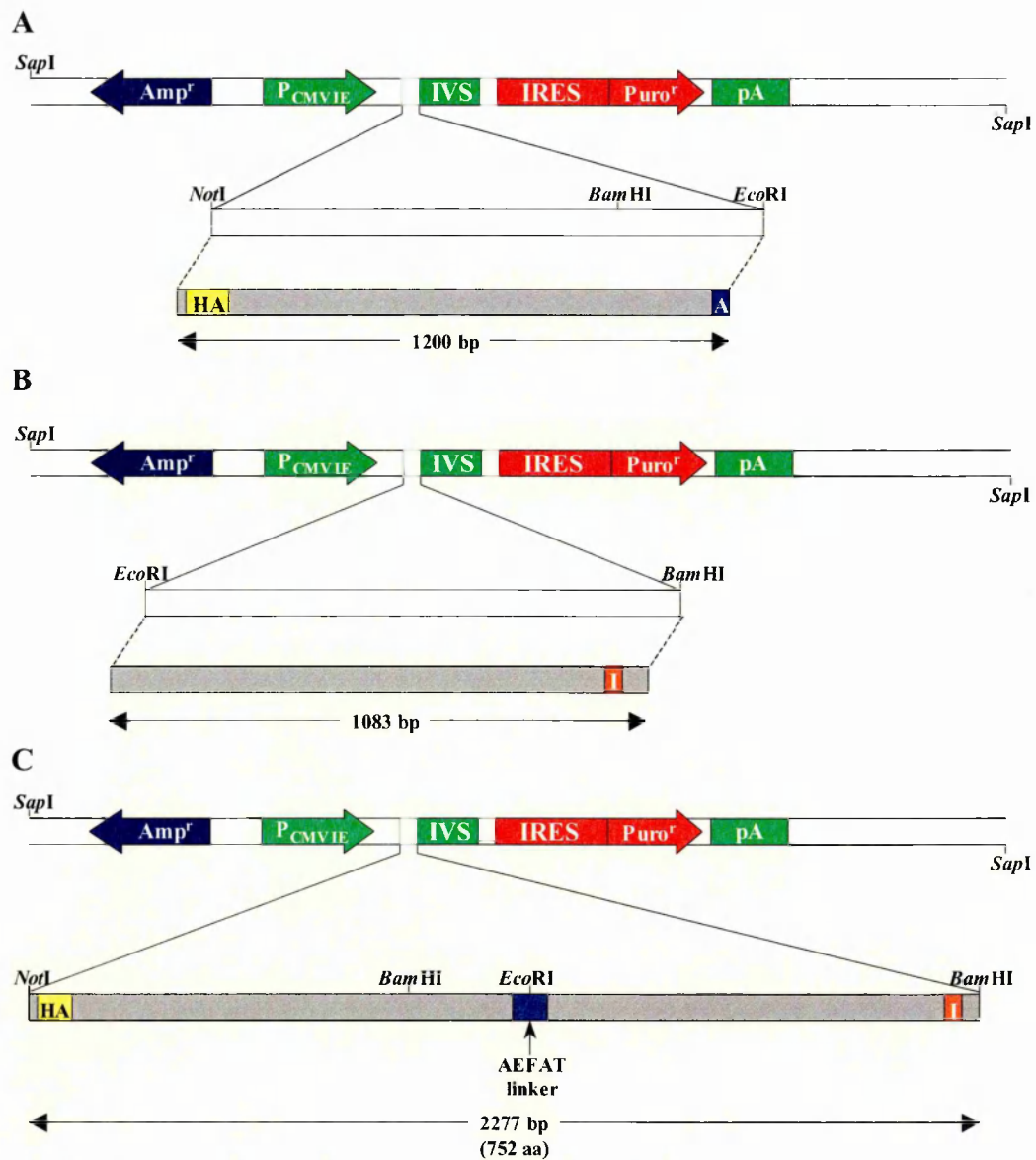
The in-frame fusion between HA-S1P₄(No Stop) and $G\alpha_{i1}(C^{351}I)$ was generated via restriction digest and three-way ligation. The 1200 bp HA-S1P₄(No Stop) insert was released by *Not*I/*Eco*RI digest of the HA-S1P₄(No Stop)/pIRES

construct (Figure 3.7 (A)), whilst the $G\alpha_{i1}(C^{351}I)/pIRES$ plasmid was digested with *EcoRI* and *BamHI* to yield the $G\alpha_{i1}(C^{351}I)$ insert, which was approximately 1083 bp in size (Figure 3.7 (B)). These two fragments were gel-purified and combined with pIRES, which had been cut with *NotI* and *BamHI* restriction enzymes, in a three-way ligation reaction, which was transformed into competent *E. Coli* bacteria. Single colonies were cultured and plasmid DNA extracted. Analysis of this DNA by restriction digest and gel electrophoresis confirmed the presence of the expected insert and one of the clones was designated HA-S1P₄- $G\alpha_{i1}(C^{351}I)/pIRES$ (Figure 3.7 (C)). The use of this cloning strategy resulted in the addition of a five amino acid artificial linker between the two elements of the fusion, which is shown in Figure 3.7 (C).

3.2.3 Generation of HA-tagged S1P₄ construct for inducible expression

In order to control the level of HA-S1P₄ expression in mammalian cells, a construct was made for use with the ecdysone-inducible mammalian expression system (Invitrogen Ltd.).

The insert of the HA-S1P₄/pIRES construct was modified by PCR such that the 5' end of the sequence contained an *EcoRI* restriction site, and a *NotI* site was present at the 3' end. These restriction sites were present in the forward and reverse oligonucleotides, 110A and 111A, respectively (see Figure 3.8 (A)). The PCR product was digested with the appropriate restriction enzymes and gel-purified before being ligated into the pIND vector, which had also been digested with *EcoRI* and *NotI* restriction enzymes. Chemically competent *E. Coli* were transformed and single colonies cultured before plasmid DNA was purified and

**Figure 3. 7****Construction of HA-S1P₄-Gα_{i1}(C³⁵¹I)/pIRES.**

The insert of the HA-S1P₄(No Stop)/pIRES construct was released by digest using *NotI* and *EcoRI* restriction enzymes (A). Fragment (B) was produced by restriction digest of the Gα_{i1}(C³⁵¹I)/pIRES with *EcoRI* and *BamHI* enzymes. Ligation of these fragments into pIRES, which had been digested with *NotI* and *BamHI* restriction enzymes, produced HA-S1P₄-Gα_{i1}(C³⁵¹I)/pIRES (C).

analysed by restriction digest and gel electrophoresis to confirm the presence of the expected insert. The insert of a single clone was sequenced over both strands, using a combination of the previously designed S1P₄-specific and

vector-specific sequencing primers. This clone was error free and was designated HA-S1P₄/pIND (shown in Figure 3.8 (B)).

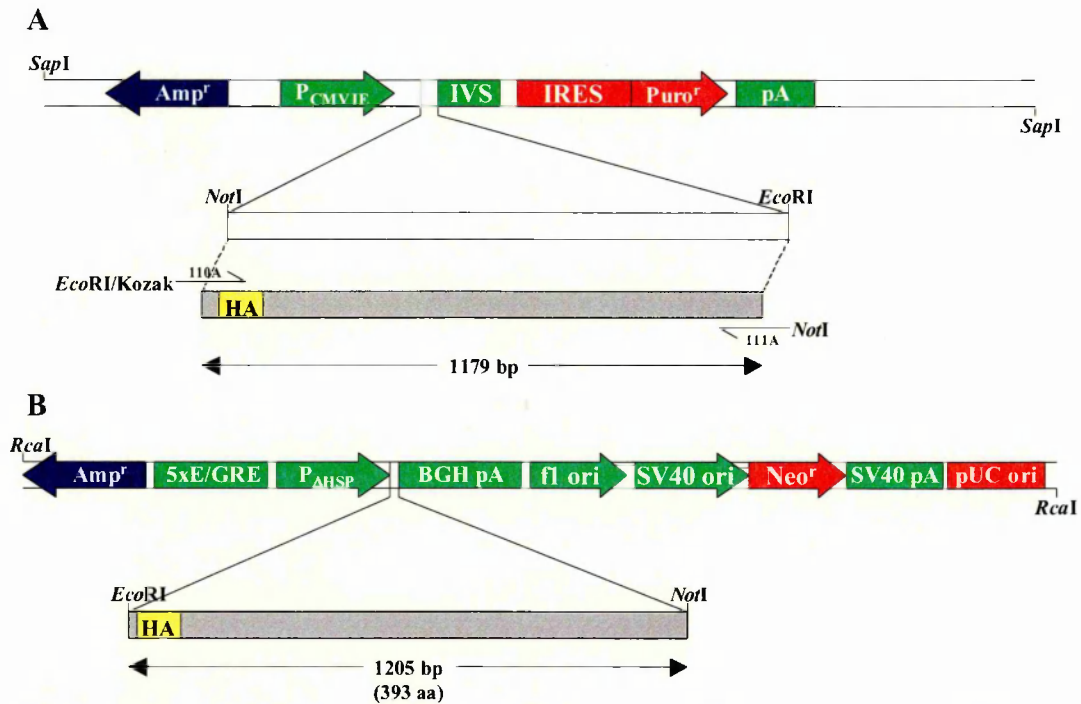


Figure 3. 8

Construction of HA-S1P₄/pIND.

PCR was used to modify the restriction sites at the 5' and 3' ends of the HA-S1P₄ insert such that they were compatible with the pIND vector. The forward oligonucleotide, 110A contained a partial Kozak sequence and introduced an *EcoRI* restriction site prior to the initiator methionine; the reverse oligonucleotide, 111A contained a *NotI* site after the stop codon of HA-S1P₄. The HA-S1P₄/pIRES construct was used as a template and the resultant PCR product (A) was digested with *EcoRI* and *NotI* restriction enzymes. Ligation into pIND, which had also been digested with *EcoRI* and *NotI* produced the construct named HA-S1P₄/pIND (B).

3.2.4 Generation of HA-tagged S1P₄(E¹²²Q) mutant fused with pertussis toxin-insensitive Gα_{i1}(C³⁵¹I)

The E¹²²Q mutation was introduced into HA-S1P₄ using PCR. Two complementary oligonucleotides, 58A and 59A, were designed across residue ¹²². Each oligonucleotide contained the necessary base modifications to mutate glutamic acid ¹²² to glutamine. Parallel PCR reactions were set up, each using

HA-S1P₄/pIRES as template DNA. The first reaction used oligonucleotides F12582 and 59A to produce a fragment of approximately 405 bp; the second reaction was performed using oligonucleotides 58A and F7241 and yielded a fragment of approximately 795 bp. After gel purification, equimolar amounts of these PCR products were mixed and a further reaction performed, using oligonucleotides F12582 and F7241. This product was gel purified then digested of with *NotI* and *NcoI* restriction enzymes, gel purified to yield the 5' fragment of approximately 476 bp, which included the desired mutation (Figure 3.9 (A)). Digestion of the previously produced HA-S1P₄(No Stop) construct with *NcoI* and *EcoRI* restriction enzymes was followed by gel purification and yielded a 3' fragment, of approximately 727 bp (Figure 3.9 (B)). The G α_{i1} (C³⁵¹I)/pIRES plasmid (produced in this chapter, section 2.2) was digested with *EcoRI* and *BamHI* to yield the G α_{i1} (C³⁵¹I) insert, which was approximately 1083 bp (Figure 3.9 (C)). An in-frame fusion between the E¹²²Q mutant of HA-S1P₄ and G α_{i1} (C³⁵¹I) was generated via four-way ligation of these fragments into *NotI/EcoRI*-digested pIRES. Single colonies of transformed *E. Coli* were cultured and restriction digestion followed by gel electrophoresis used to check for the presence of insert in the purified plasmid DNA. The HA-S1P₄ insert of a single clone was sequenced on both strands and was found to contain the desired mutation and to be free from PCR errors. This construct was named HA-S1P₄(E¹²²Q)-G α_{i1} (C³⁵¹I)/pIRES and is shown in Figure 3.9 (D). In common with the wild type HA-S1P₄-G α_{i1} (C³⁵¹I) fusion protein, HA-S1P₄(E¹²²Q)-G α_{i1} (C³⁵¹I) contained an artificial five amino acid linker present between the two elements of the fusion protein, which was a consequence of the cloning strategy used.

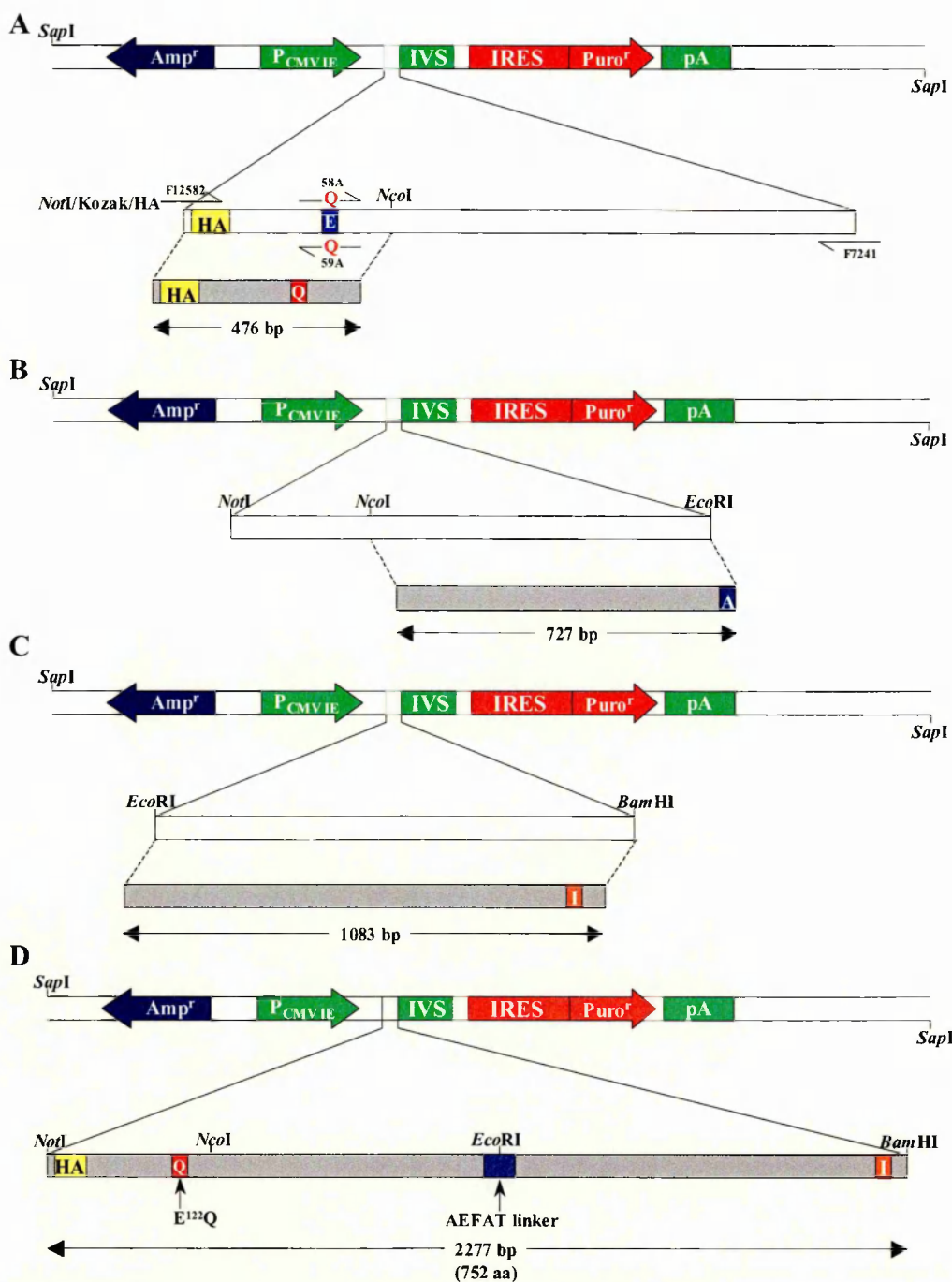


Figure 3. 9
Construction of HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I)/pIRES.
Mutation of glutamic acid at position ¹²² of S1P₄ to glutamine was achieved by parallel PCR reactions. The 5' portion of the sequence was amplified using the oligonucleotides F12582 (forwards) and 59A (reverse), whilst the forward oligonucleotide 58A and the reverse oligonucleotide F7241 were used to amplify the 3' portion of the sequence. The PCR products were combined and a further amplification performed using the oligonucleotides F12582 and F7241. This product was digested with *NotI* and *NcoI* restriction enzymes (A). Other fragments to produce this construct were generated by restriction digest: (B) 3' *NcoI*/*EcoRI* fragment of HA-S1P₄(No Stop) and (C) *EcoRI*/*BamHI* insert from Gα_{i1}(C³⁵¹I)/pIRES. These fragments were ligated into *NotI*/*BamHI* digested pIRES to yield HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I)/pIRES (D).

3.2.5 Determination of expression of endogenous S1P and LPA receptors in CHO-K1 cells by RT-PCR

Untransfected CHO-K1 cells respond to exogenous S1P in a variety of *in-vitro* assays, which is thought to be due to the expression of endogenous S1P receptors by these cells. Although several mammalian cell lines have been characterised with respect to expression of endogenous S1P and LPA (Edg) receptors (examples in Graeler *et al.*, 1998; Motohashi *et al.*, 2000; Fueller *et al.*, 2003), very little data of this nature has been reported for the CHO-K1 host. This may be due to this cell line being derived from Chinese hamster. Very few gene sequences have been published for this species and of those which had been deposited at the time of the current investigation, none were of S1P or LPA receptors.

Such a lack of sequence data meant that the design of oligonucleotides for use in RT-PCR experiments to determine which of the S1P and LPA receptors were endogenously expressed by CHO-K1 cells was not straightforward. Human and rodent sequences for each of the eight Edg receptors were aligned, both at the nucleotide and protein level, and areas of high similarity between species were identified as the optimal areas over which oligonucleotides could be designed. It was noted that, for each of the Edg receptors, there was a strong degree of conservation between species at the protein level, although this was less obvious in nucleotide sequences since there were often changes at the 3-position between different species, which were silent with respect to the protein sequence. Hence, it was necessary to design primers which contained a degree of redundancy. The LPA receptors were known to contain an intron in TM VI, the location of which is absolutely conserved between human and mouse [Contos and Chun, 1998;

Contos and Chun, 2000; Contos and Chun, 2001]. Oligonucleotides to amplify a fragment of each of the LPA receptor genes were designed such that this intron boundary was not spanned by the expected product. Table 3.1 shows the identity of the forward and reverse primers designed to amplify each of the S1P and LPA receptor genes from Chinese hamster.

	Forward oligonucleotide	Reverse oligonucleotide	Expected fragment (bp)
S1P ₁	1170B	1171B	440
S1P ₂	1176B	1177B	560
S1P ₃	1172B	1173B	610
S1P ₄	53B	54B	485
S1P ₅	1182B	58B	625
LPA ₁	45B	46B	610
LPA ₂	49B	1174B	590
LPA ₃	1178B	1180B	720

Table 3. 1

Oligonucleotides used to clone fragments of S1P and LPA receptor genes from Chinese hamster genomic DNA.

Oligonucleotides were tested using genomic DNA prepared from CHO-K1 cells as template in PCR reactions (Figure 3.10); products of the expected sizes were obtained for each primer pair. PCR products were cloned into the TOPO TA Cloning Kit for Sequencing vector and transformed into TOP10 chemically competent *E. Coli*. Plasmid DNA was purified from cultures grown from single colonies and digested with *EcoRI* to confirm the presence of an insert. Vector-specific primers were used to sequence the entire insert of four clones of each receptor construct and the consensus sequence obtained for each Chinese hamster gene fragment aligned with known human and rodent sequences for that gene, at both the nucleotide and protein levels. Alignments of translated partial

Chinese hamster gene fragment sequences with translated coding sequences of human, mouse and, where available, rat genes are shown in Figure 3.11 (A-H).

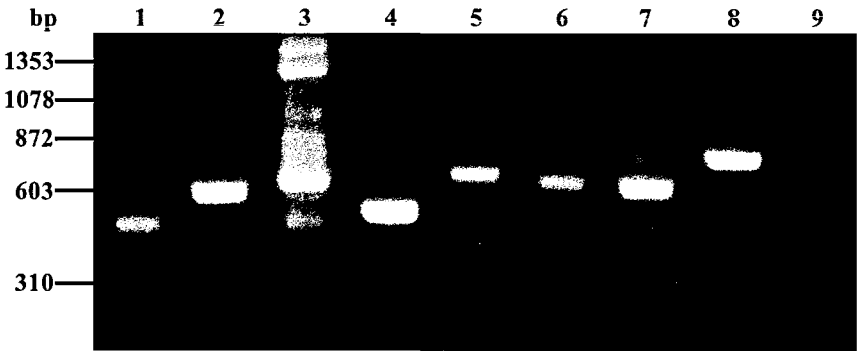


Figure 3. 10
PCR amplification of partial S1P and LPA receptor sequences from Chinese hamster genomic DNA.
Oligonucleotides to amplify a fragment of each Chinese hamster S1P and LPA receptor were designed using sequences of human, mouse and, where available, rat S1P and LPA receptors. Genomic DNA was prepared from CHO-K1 cells and used as template in PCR reactions with these oligonucleotides pairs and the PCR products analysed using gel electrophoresis. This Figure is representative of three independent experiments.

Lane 1	S1P ₁
Lane 2	S1P ₂
Lane 3	S1P ₃
Lane 4	S1P ₄
Lane 5	S1P ₅
Lane 6	LPA ₁
Lane 7	LPA ₂
Lane 8	LPA ₃
Lane 9	LPA ₃ (no DNA)

A

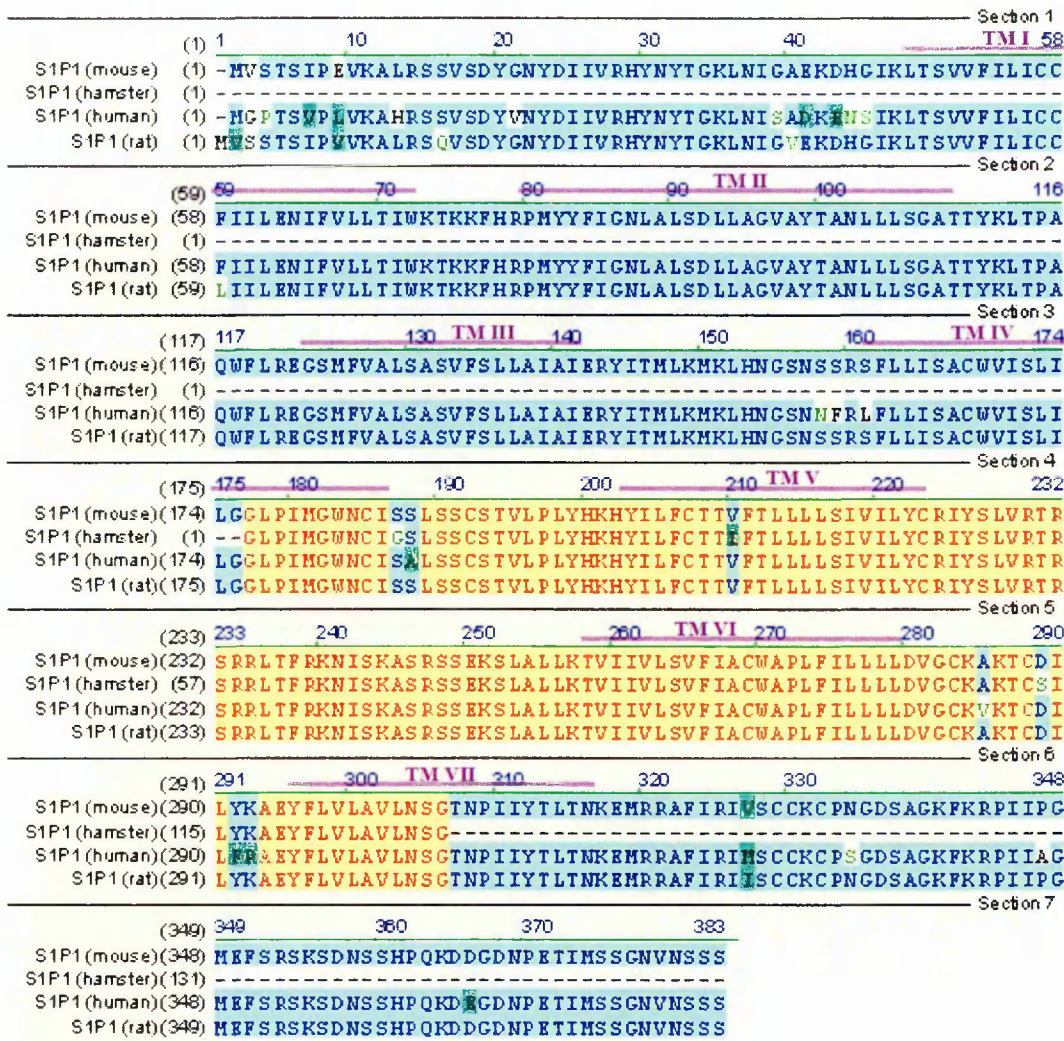


Figure 3. 11
Alignment of human, mouse and rat S1P and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA.

Amplification of a portion of the coding sequence of each of the S1P and LPA receptors present in the genome of the Chinese hamster was achieved via PCR using oligonucleotide pairs which had been designed using alignments of human, mouse and, where available, rat S1P and LPA receptor sequences. The products of these PCR reactions were cloned and sequenced. The translated sequence was aligned with protein sequences of human and rodent S1P and LPA receptors and the alignment annotated to show the location of each transmembrane (TM) domain.

A S1P₁ (Edg1)

B

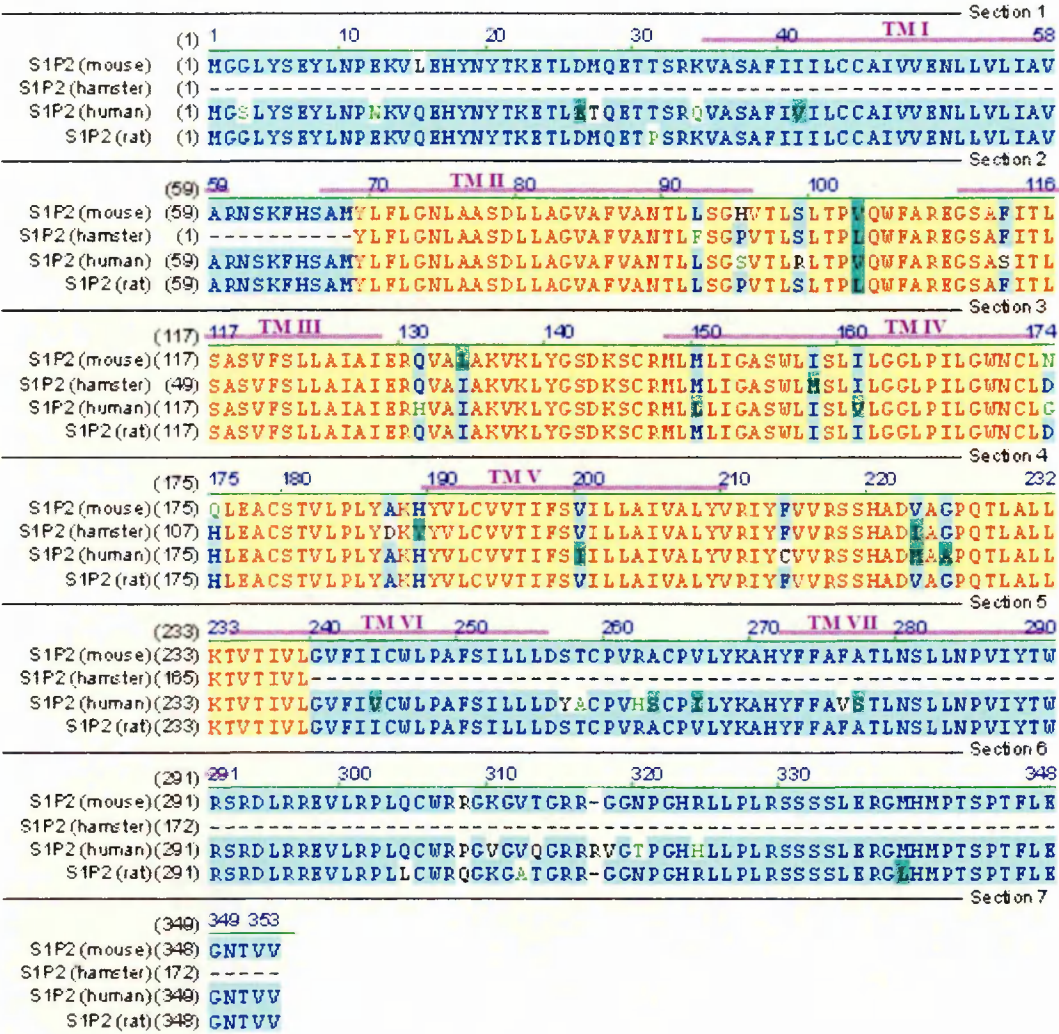


Figure 3. 11 (continued)
Alignment of human, mouse and rat S1P and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA.

Amplification of a portion of the coding sequence of each of the S1P and LPA receptors present in the genome of the Chinese hamster was achieved via PCR using oligonucleotide pairs which had been designed using alignments of human, mouse and, where available, rat S1P and LPA receptor sequences. The products of these PCR reactions were cloned and sequenced. The translated sequence was aligned with protein sequences of human and rodent S1P and LPA receptors and the alignment annotated to show the location of each transmembrane (TM) domain.

B S1P₂ (Edg5)

C

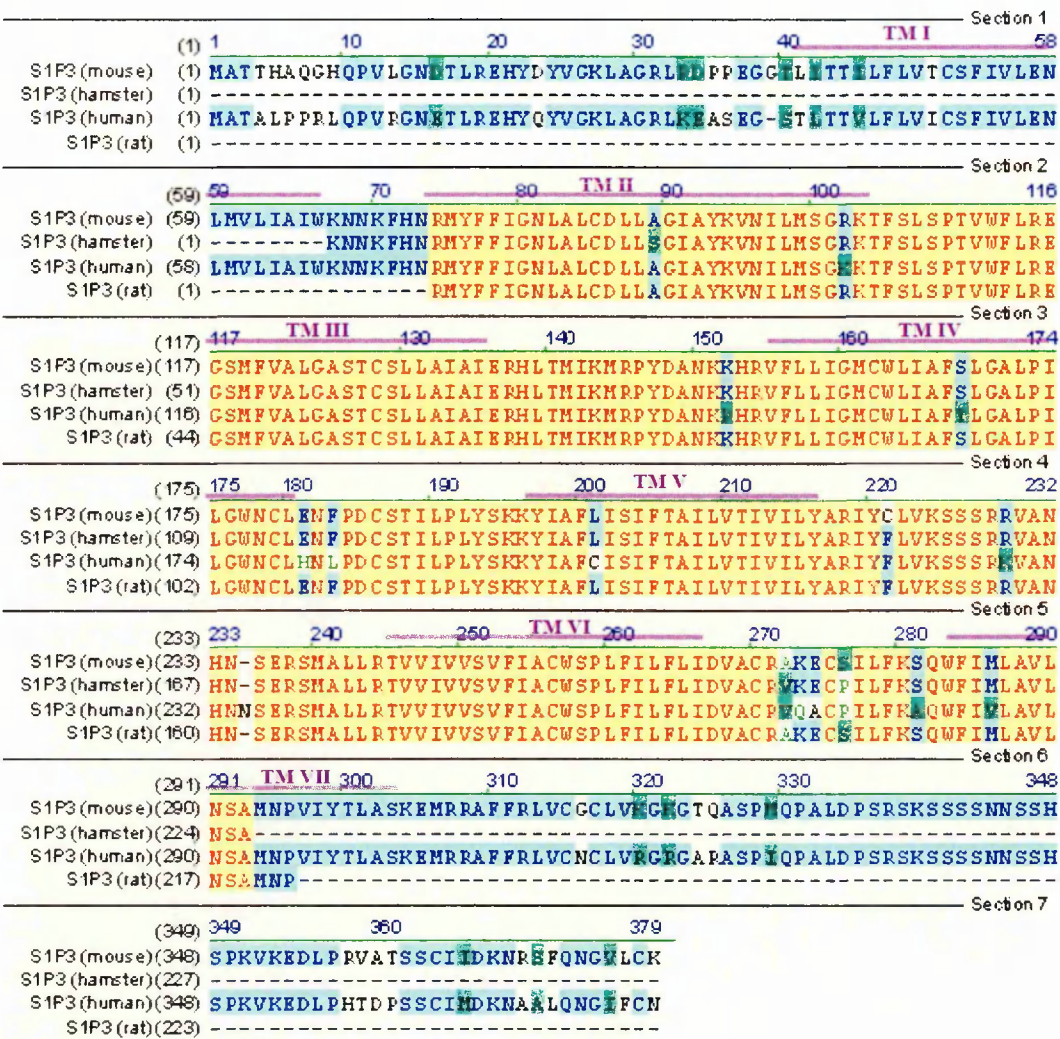


Figure 3. 11 (continued)
Alignment of human, mouse and rat S1P and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA.

Amplification of a portion of the coding sequence of each of the S1P and LPA receptors present in the genome of the Chinese hamster was achieved via PCR using oligonucleotide pairs which had been designed using alignments of human, mouse and, where available, rat S1P and LPA receptor sequences. The products of these PCR reactions were cloned and sequenced. The translated sequence was aligned with protein sequences of human and rodent S1P and LPA receptors and the alignment annotated to show the location of each transmembrane (TM) domain.

C S1P₃ (Edg3)

D

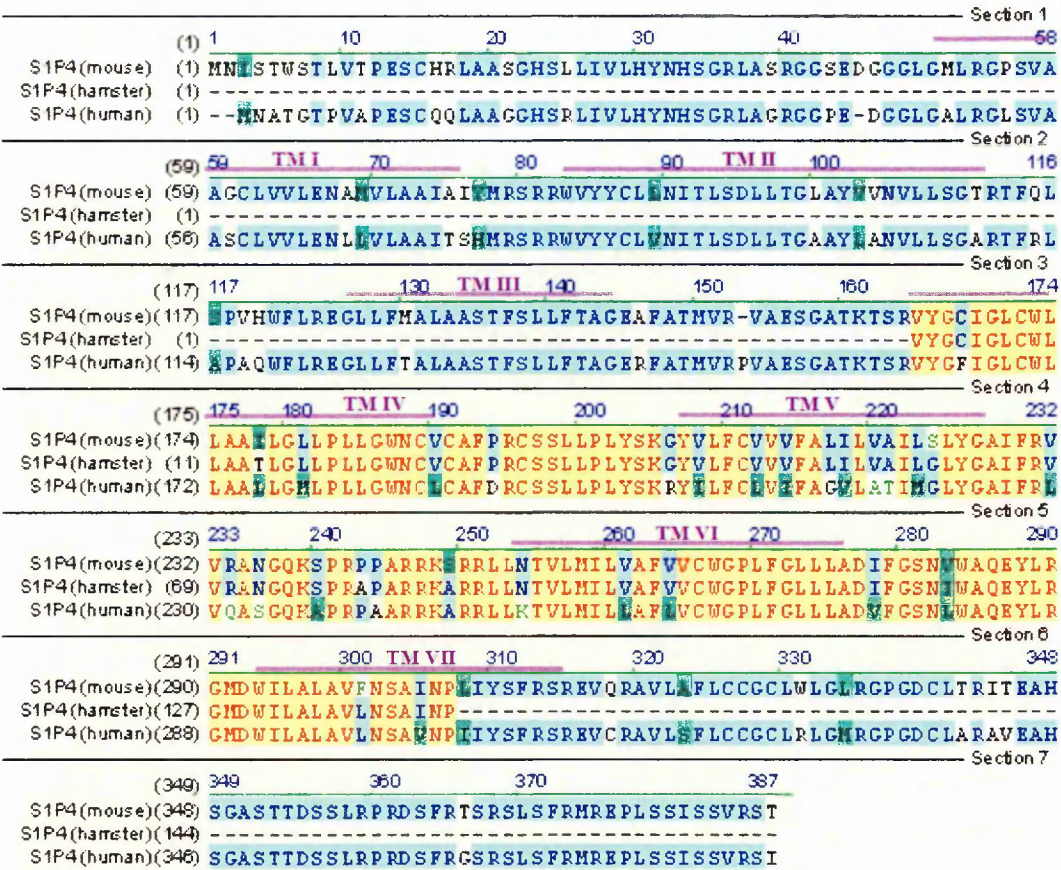


Figure 3. 11 (continued)
Alignment of human, mouse and rat S1P and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA.

Amplification of a portion of the coding sequence of each of the S1P and LPA receptors present in the genome of the Chinese hamster was achieved via PCR using oligonucleotide pairs which had been designed using alignments of human, mouse and, where available, rat S1P and LPA receptor sequences. The products of these PCR reactions were cloned and sequenced. The translated sequence was aligned with protein sequences of human and rodent S1P and LPA receptors and the alignment annotated to show the location of each transmembrane (TM) domain.

D S1P₄ (Edg6)

E

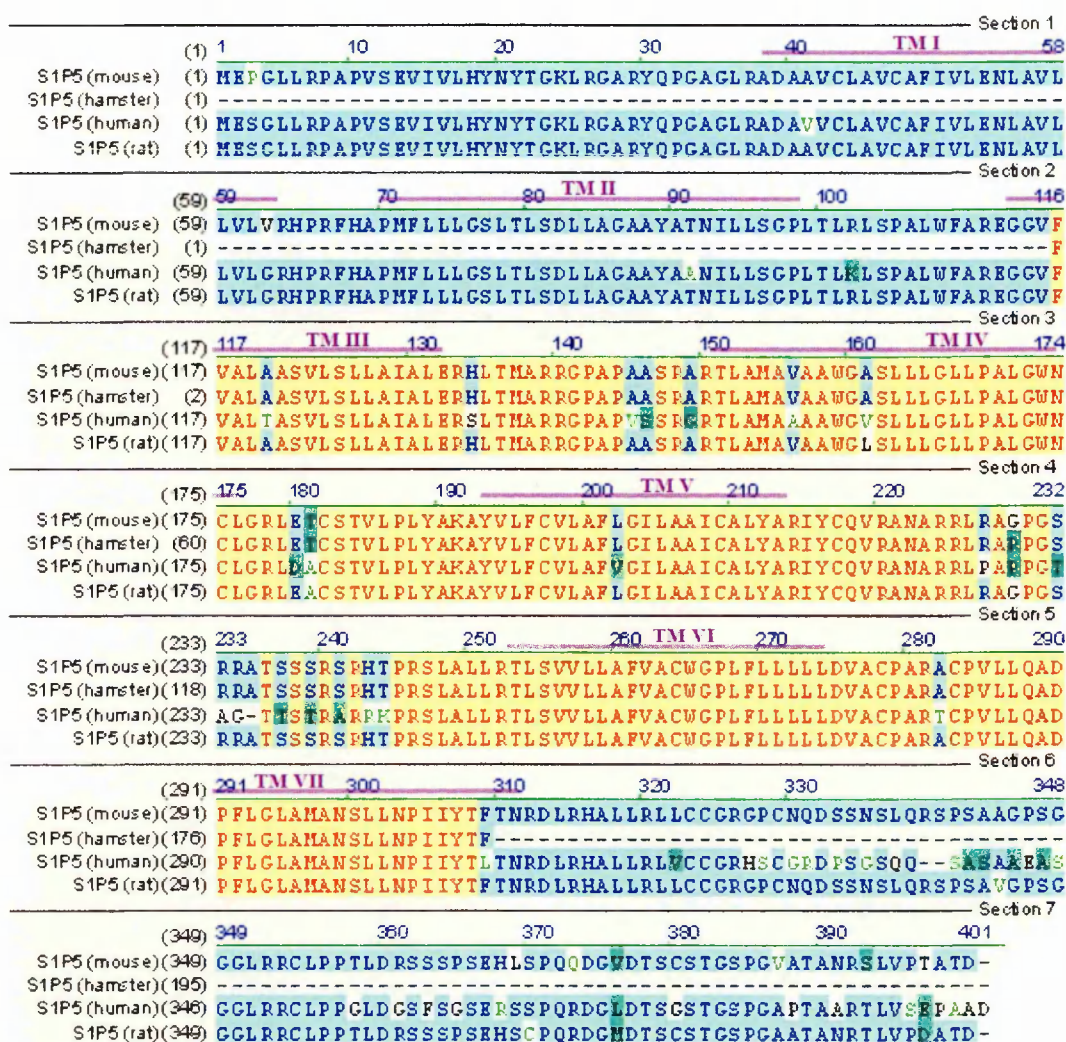


Figure 3. 11 (continued)

Alignment of human, mouse and rat S1P and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA.

Amplification of a portion of the coding sequence of each of the S1P and LPA receptors present in the genome of the Chinese hamster was achieved via PCR using oligonucleotide pairs which had been designed using alignments of human, mouse and, where available, rat S1P and LPA receptor sequences. The products of these PCR reactions were cloned and sequenced. The translated sequence was aligned with protein sequences of human and rodent S1P and LPA receptors and the alignment annotated to show the location of each transmembrane (TM) domain.

E S1P₅ (Edg8)

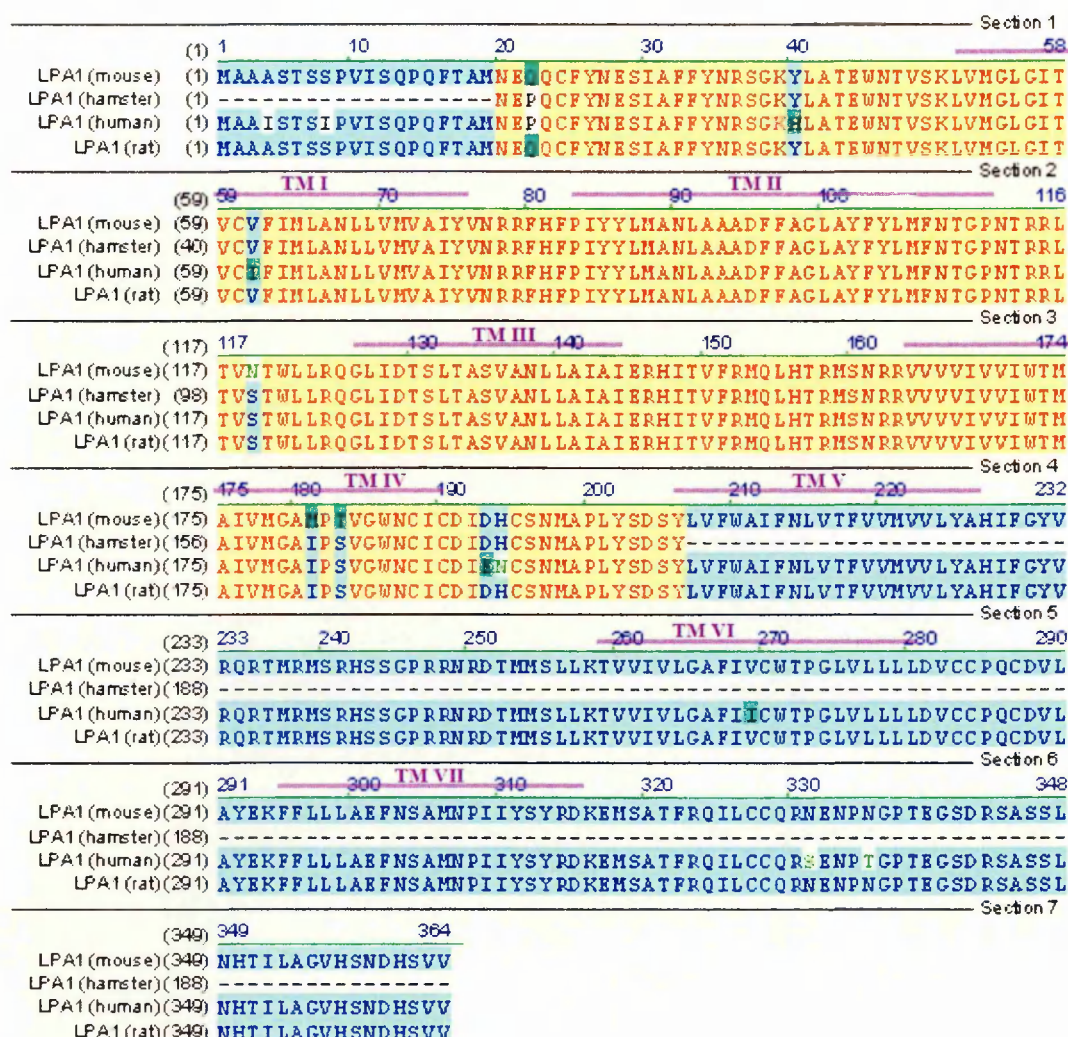
F

Figure 3. 11 (continued)

Alignment of human, mouse and rat S1P and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA.

Amplification of a portion of the coding sequence of each of the S1P and LPA receptors present in the genome of the Chinese hamster was achieved via PCR using oligonucleotide pairs which had been designed using alignments of human, mouse and, where available, rat S1P and LPA receptor sequences. The products of these PCR reactions were cloned and sequenced. The translated sequence was aligned with protein sequences of human and rodent S1P and LPA receptors and the alignment annotated to show the location of each transmembrane (TM) domain.

F LPA₁ (Edg2)

G

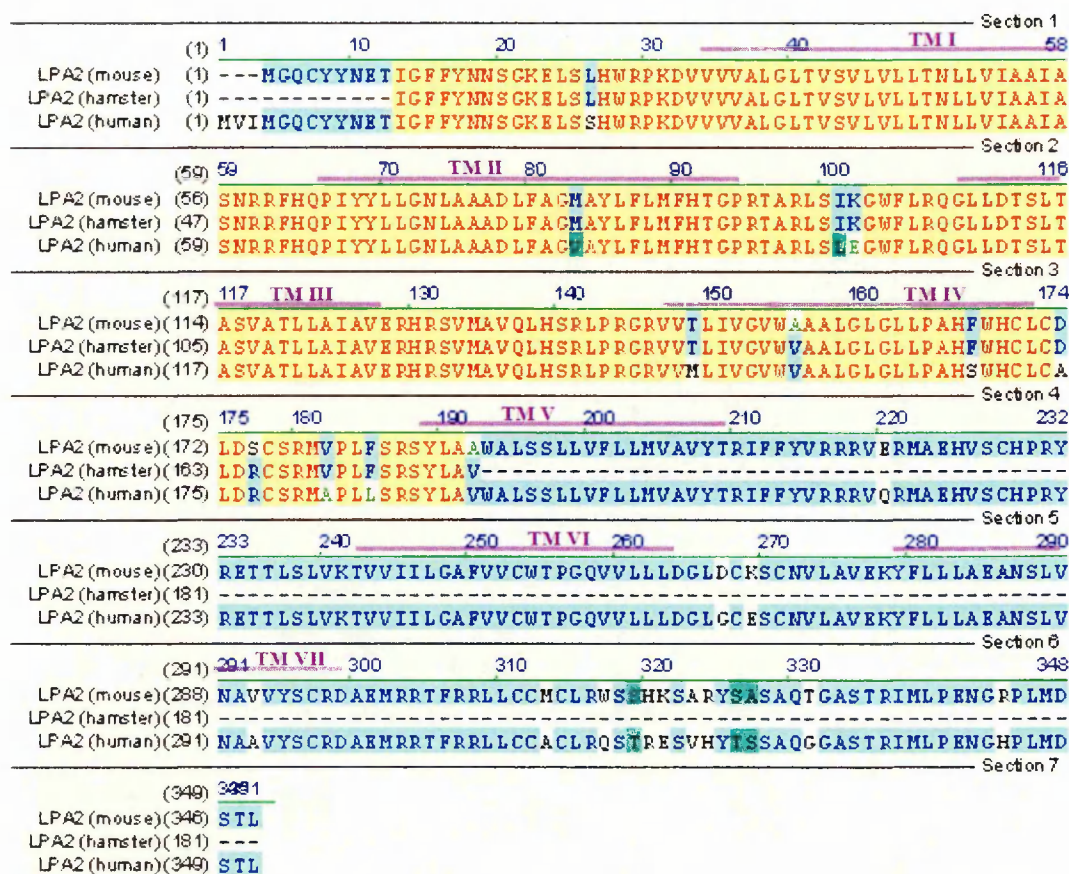


Figure 3. 11 (continued)

Alignment of human, mouse and rat S1P and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA.

Amplification of a portion of the coding sequence of each of the S1P and LPA receptors present in the genome of the Chinese hamster was achieved via PCR using oligonucleotide pairs which had been designed using alignments of human, mouse and, where available, rat S1P and LPA receptor sequences. The products of these PCR reactions were cloned and sequenced. The translated sequence was aligned with protein sequences of human and rodent S1P and LPA receptors and the alignment annotated to show the location of each transmembrane (TM) domain.

G LPA₂ (Edg4)

H

		Section 1																																																							
	(1)	1	10	20	30	40	TMI			58																																															
LPA3 (mouse)	(1)	MNECHYDKRMDFFYNRSNTDTADEWETGTRKLVIVLCVGTFFCLFIFFSNSLVIAAVITN																																																							
LPA3 (hamster)	(1)	-----DFFYNRSNTDTADEWETGTRKLVIVLCVGTFFCLFIFFSNSLVIAAVITN																																																							
LPA3 (human)	(1)	MNECHYDKRMDFFYNRSNTDTADEWETGTRKLVIVLCVGTFFCLFIFFSNSLVIAAVIKN																																																							
LPA3 (rat)	(1)	MNECHYDKRMDFFYNRSNTDTADEWETGTRKLVIVLCVGTFFCLFIFFSNSLVIAAVITN																																																							
		Section 2																																																							
	(59)	59	70	80	90	100	TMII			116																																															
LPA3 (mouse)	(59)	RRKFHPPFYLLANLAAADFFAGIAYVFLMFNTGPVSKTLTVNRWFLRQGLLDISLTAS																																																							
LPA3 (hamster)	(49)	RRKFHPPFYLLANLAAADFFAGIAYVFLMFNTGPVSKTLTVNRWFLRQGLLDISLTAS																																																							
LPA3 (human)	(59)	RRKFHPPFYLLANLAAADFFAGIAYVFLMFNTGPVSKTLTVNRWFLRQGLLDISLTAS																																																							
LPA3 (rat)	(59)	RRKFHPPFYLLANLAAADFFAGIAYVFLMFNTGPVSKTLTVNRWFLRQGLLDISLTAS																																																							
		Section 3																																																							
	(117)	117	130	140	150	160	TMIII			174																																															
LPA3 (mouse)	(117)	LANLLVIAVERHMSIMRMVHSNLTKKRVTLLILLVWAIATFMCAVPTLGWNCNLCNIS																																																							
LPA3 (hamster)	(107)	LANLLVIAVERHMSIMRMVHSNLTKKRVTLLILLVWAIATFMCAVPTLGWNCNLCNIS																																																							
LPA3 (human)	(117)	LNLVIAVERHMSIMRMVHSNLTKKRVTLLILLVWAIATFMCAVPTLGWNCNLCNIS																																																							
LPA3 (rat)	(117)	LANLLVIAVERHMSIMRMVHSNLTKKRVTLLILLVWAIATFMCAVPTLGWNCNLCNIS																																																							
		Section 4																																																							
	(175)	175	180	190	200	210	220	TMV			232																																														
LPA3 (mouse)	(175)	ACSSLAPIYSRSYLI FWTVSNLLAFFIMHVVYVRIYMYVKRKTNVLS PHTSGSISR																																																							
LPA3 (hamster)	(165)	ACSSLAPIYSRSYLI FWTVSNLLAFFIMHVVYVRIYMYVKRKTNVLS PHTSGSISR																																																							
LPA3 (human)	(175)	ACSSLAPIYSRSYLI FWTVSNLLAFFIMHVVYVRIYMYVKRKTNVLS PHTSGSISR																																																							
LPA3 (rat)	(175)	ACSSLAPIYSRSYLI FWTVSNLLAFFIMHVVYVRIYMYVKRKTNVLS PHTSGSISR																																																							
		Section 5																																																							
	(233)	233	240	250	260	270	280	TMVI			290																																														
LPA3 (mouse)	(233)	APMKLMKTVMITVLGAFVVCWTPGLVLLLDGLNCKQCNVQHVSRWFLLLALLNSVMNP																																																							
LPA3 (hamster)	(223)	APMKLMKTVMITVLGAFVVCWTPGLVLLLDGLNCKQCNVQHVSRWFLLLALLNSVMNP																																																							
LPA3 (human)	(233)	APMKLMKTVMITVLGAFVVCWTPGLVLLLDGLNCKQCNVQHVSRWFLLLALLNSVMNP																																																							
LPA3 (rat)	(233)	APMKLMKTVMITVLGAFVVCWTPGLVLLLDGLNCKQCNVQHVSRWFLLLALLNSVMNP																																																							
		Section 6																																																							
	(291)	291	300	310	320	330	TMVII			348																																															
LPA3 (mouse)	(291)	IIYSYKDEDMYNTHRMKMICCALQDSNTERRPSRNPSTIHSRSITGSQYLEDSISQGPV																																																							
LPA3 (hamster)	(225)	IIYSYKDEDMYNTHRMKMICCFSGE-NPERRPSRNPSTIHSRSITGSQYLEDSISQGV																																																							
LPA3 (human)	(291)	IIYSYKDEDMYNTHRMKMICCAPHDSNAERHPSRNPSTIHSRSITGSQYLEDSISQGV																																																							
LPA3 (rat)	(291)	IIYSYKDEDMYNTHRMKMICCAPHDSNAERHPSRNPSTIHSRSITGSQYLEDSISQGV																																																							
		Section 7																																																							
	(349)	349	354																																																						
LPA3 (mouse)	(349)	CNKNGS																																																							
LPA3 (hamster)	(225)	CNKSTS																																																							
LPA3 (human)	(348)	CNKSTS																																																							
LPA3 (rat)	(349)	CNKSSS																																																							

Figure 3. 11 (continued)

Alignment of human, mouse and rat S1P and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA.

Amplification of a portion of the coding sequence of each of the S1P and LPA receptors present in the genome of the Chinese hamster was achieved via PCR using oligonucleotide pairs which had been designed using alignments of human, mouse and, where available, rat S1P and LPA receptor sequences. The products of these PCR reactions were cloned and sequenced. The translated sequence was aligned with protein sequences of human and rodent S1P and LPA receptors and the alignment annotated to show the location of each transmembrane (TM) domain.

H LPA₃ (Edg7)

Both nucleotide and protein sequences of the partial Chinese hamster sequences were found to be very similar to those from human, rat and mouse. Percent identities were calculated for novel Chinese hamster sequences compared to human, mouse or rat orthologues using pairwise comparisons of sequences at the nucleotide (Table 3.2 (A)) and translated protein level (Table 3.2 (B)).

A

	% identity		
	Human	Mouse	Rat
S1P ₁	88.7	95.0	94.6
S1P ₂	82.6	90.6	92.5
S1P ₃	89.5	93.1	94.0
S1P ₄	83.3	91.1	*
S1P ₅	81.9	91.0	90.7
LPA ₁	90.9	93.2	93.7
LPA ₂	86.1	92.0	*
LPA ₃	87.3	90.1	90.3

B

	% identity		
	Human	Mouse	Rat
S1P ₁	94.6	97.7	97.7
S1P ₂	90.6	94.2	97.1
S1P ₃	95.2	98.9	99.4
S1P ₄	81.8	95.8	*
S1P ₅	88.7	99.5	98.5
LPA ₁	97.9	97.9	99.5
LPA ₂	95.0	98.3	*
LPA ₃	93.3	97.8	98.2

Table 3. 2

Summary of similarities between cloned partial Chinese hamster S1P and LPA receptor sequences and those from mouse, rat and human.

Pairwise comparisons of the cloned Chinese hamster sequence of each S1P and LPA receptor gene fragment with the mouse, rat or human orthologues were performed and the percentage identity determined for the range of the partial Chinese hamster sequence.

* - sequence not available from National Centre for Biotechnology Information (NCBI) Entrez database.

A Summary of nucleotide sequence identities

B Summary of protein sequence identities

The cloned Chinese hamster gene fragments shared a high degree of identity with their mouse, rat and human orthologues, showing between 81.9 and 95.0 % identity at the nucleotide level and between 81.8 and 99.5 % at the protein level. In general, the level of identity was higher for the protein sequence than the nucleotide sequence for a given receptor gene, although this was not the case for the comparison between human and hamster S1P₄ (where the level of identity at the nucleotide level was slightly higher than at the protein level). It was observed that many differences in the nucleotide sequences of a given S1P or LPA receptor gene from each of the species examined, occurred at the third position of a codon and hence were not observed as a difference in the protein sequence. Chinese hamster S1P and LPA receptor gene fragments always shared greater sequence identity with mouse and rat orthologues than with human.

Each Chinese hamster gene consensus sequence was then used to facilitate design of non-redundant oligonucleotides for use in the RT-PCR experiment.

The identity of these oligonucleotides is shown in Table 3.3.

	Forward oligonucleotide	Reverse oligonucleotide	Expected fragment (bp)
S1P ₁	1364C	1365C	380
S1P ₂	1372C	1373C	510
S1P ₃	1368C	1369C	560
S1P ₄	1374C	1375C	425
S1P ₅	1378C	1379C	580
LPA ₁	1366C	1367C	560
LPA ₂	1370C	1371C	540
LPA ₃	1376C	1377C	670

Table 3. 3

Oligonucleotides used for RT-PCR analysis of CHO-K1 mRNA to identify endogenously expressed S1P and LPA receptor genes.

Total RNA was prepared from CHO-K1 cells and treated to remove contaminating genomic DNA (described in chapter 2, section 2.16). Reverse transcription using an oligo dT₍₁₈₎ primer yielded first strand cDNA which was then used as template in PCR reactions with the oligonucleotide pairs shown in Table 3.3 to determine which S1P and LPA receptor mRNA's were expressed in CHO-K1 cells (Figure 3.13 (A)). The ability of each oligonucleotide pair to amplify a product of the expected size was demonstrated using CHO-K1 genomic DNA (Figure 3.13 (B)) and the integrity of the first strand cDNA preparation was verified using control GAPDH oligonucleotides. The absence of DNA contamination in the RNA preparation was confirmed by the inability of the primer pairs to amplify products when the reverse transcription step was omitted (not shown) and the identity of each amplified S1P and LPA receptor fragment was confirmed by size and restriction digest of that product.

Fragments of the expected sizes were amplified using oligonucleotide pairs specific for the S1P₁, S1P₂, S1P₄ and LPA₁ receptors; the control GAPDH primers also amplified a product of the expected size. The intensity of the fragments amplified in this qualitative experiment varied; the S1P₂ fragment was the most intense, fragments of the S1P₁ and LPA₁ receptors, as well as GAPDH had lower intensity but were still clearly visible, whilst the S1P₄ fragment was very faint. Although this experiment was not quantitative, this result may suggest that the transcript for S1P₄ was less abundant than those of the S1P₁, S1P₂ and LPA₁ receptor genes.

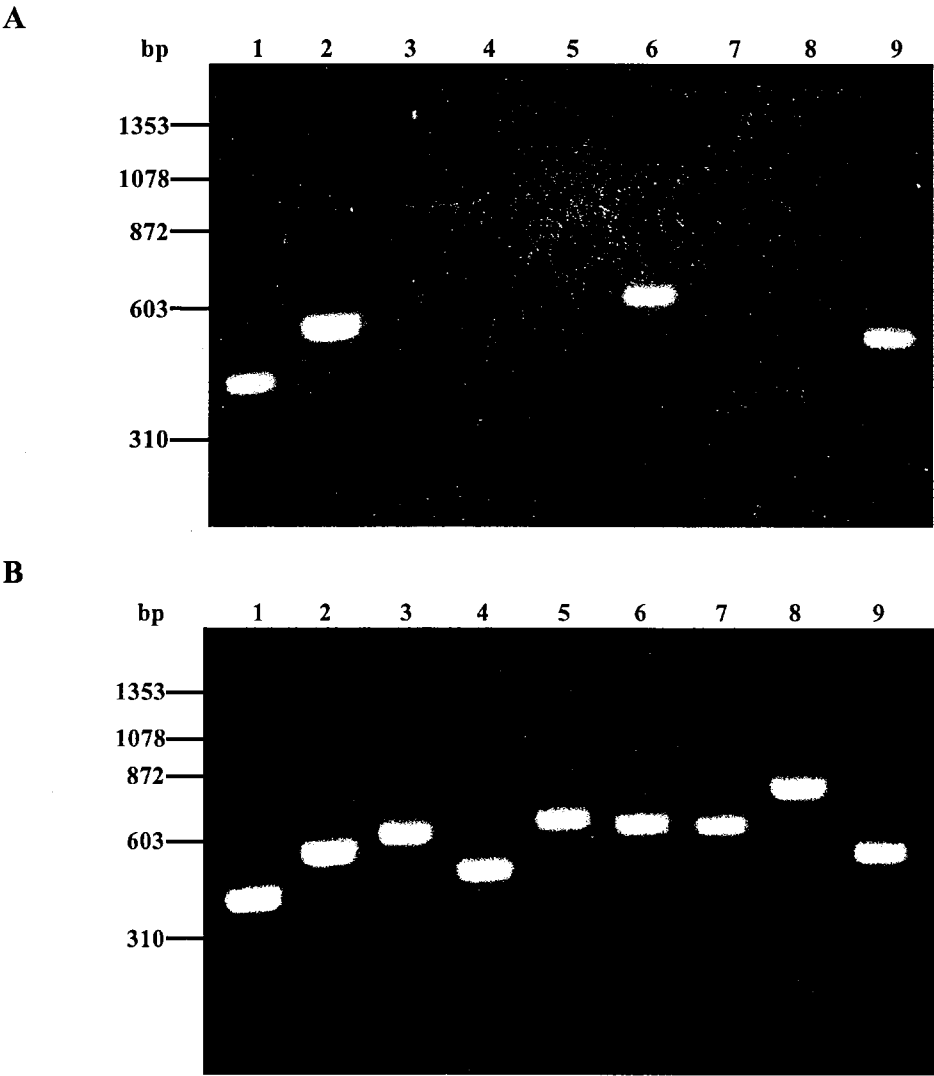


Figure 3. 12
Expression of S1P and LPA receptor mRNA in CHO-K1 cells by RT-PCR.
RT-PCR was performed using total RNA that had been extracted from CHO-K1 cells, with pairs of oligonucleotides designed to amplify specific regions of hamster S1P and LPA receptor coding sequences (A). The ability of these primer pairs to amplify products of the expected size was confirmed using CHO-K1 genomic DNA as template in a parallel set of PCR reactions (B). 5 μ L of a 20 μ L reaction was loaded in each lane. This Figure is representative of two independent experiments.

Lane 1	S1P ₁
Lane 2	S1P ₂
Lane 3	S1P ₃
Lane 4	S1P ₄
Lane 5	S1P ₅
Lane 6	LPA ₁
Lane 7	LPA ₂
Lane 8	LPA ₃
Lane 9	GAPDH

3.3 Discussion

At the time this work was started, S1P₄ had recently been cloned as the orphan receptor Edg6 [Graeler *et al.*, 1998]. This novel sequence was discovered in an effort to clone new chemokine receptors and was assigned to the then named Edg receptor family on the basis of sequence homology with other Edg receptors. The study of an orphan receptor presents a number of challenges, one of the most obvious being the inability to use a functional method for screening antibiotic-resistant clones which result from a stable transfection of the orphan into a suitable cell line. Similarly, commercial antibodies to orphan receptors are frequently unavailable, so the use of such an antibody in a technique such as Western blotting is also prohibited. For these reasons, S1P₄ expression plasmids were engineered to contain the HA epitope (YPYDVPDYA) immediately after the initiator methionine. This allowed detection of cell-surface receptor via FACS analysis, and of full length protein via Western blotting, using an anti-HA antibody. The addition of such a tag to the amino-terminus of a GPCR has been used previously and was shown to have no effect on the ability of ligand to bind the receptor [Koller *et al.*, 1997]. In the case of the S1P₄ receptor, the ligand was expected to be S1P or a related lysophospholipid, and as such would be most likely to bind to the transmembrane helices of the receptor so the inclusion of a nine amino acid epitope at the amino-terminus was not expected to interfere with ligand binding.

The use of fusion proteins constructed between a GPCR and a G protein is a popular approach and has a number of advantages over systems where non-fused receptors are studied (reviewed by Seifert *et al.*, 1999; Milligan, 2000; Wurch and Pauwels, 2001). The key benefit of constructing such fusions include a

defined 1:1 stoichiometry between receptor and G protein, and a physical proximity between the proteins. Additionally, construction of a GPCR-G α fusion may be achieved using a G α G protein which has been mutated such that it is rendered insensitive to ADP-ribosylation by pertussis toxin [Milligan, 1988; Bahia *et al.*, 1998], therefore allowing isolation of the response from the fused receptor. This was of particular relevance when applied to a receptor such as S1P₄, since it is one of a sub-family of receptors that are activated by S1P and are almost ubiquitously expressed in mammalian cells. This has meant that functional responses by untransfected cells are commonplace and can interfere with interpretation of results generated using transfected cells. The lack of S1P receptor-selective agonists combined with widespread expression of the receptors in mammalian cells has complicated the characterisation of this GPCR family. Generation of the pertussis toxin-insensitive HA-S1P₄-G α_{i1} (C³⁵¹I) fusion protein in this chapter enabled the use of pertussis toxin to eliminate interfering signals arising from the presence of endogenous S1P receptors in the host CHO-K1 cells when measuring activation of G α_i G proteins by S1P. Indeed, a similar approach was adopted in an early study which examined activation of the related LPA₁ (Edg2) receptor in HEK-293 cells [McAllister *et al.*, 2000].

The cloning strategy adopted to produce fusions between S1P₄ and G α_{i1} resulted in the introduction of a five amino acid artificial linker sequence between the two elements of the fusion. The presence of this short linker is unlikely to impact on the ability of the receptor to signal via the tethered G protein and a published study showed that the presence of the large green fluorescent protein (GFP) in a

fusion protein between the A1 adenosine receptor, GFP and G α_{i1} had little effect on ligand binding or effector regulation by this GPCR [Bevan *et al.*, 1999].

The final section of work undertaken in this chapter was to profile the expression of endogenous S1P and LPA receptors in CHO-K1 cells. Whilst it was known that most cell types respond to S1P and LPA, and presumably express at least one receptor from each of these families, there had been no concerted study to determine the repertoire of expression of these receptors in CHO-K1 cells. Some interesting observations had been made using Northern blotting techniques, however the probes used in these studies were not hamster-gene specific, and therefore interpretation of the results needs caution [Okamoto *et al.*, 1998; Sato *et al.*, 1999].

The approach taken in this thesis was to perform RT-PCR using Chinese hamster mRNA with oligonucleotides specific to each of the eight Chinese hamster S1P and LPA gene coding sequences. Searches of the NCBI Entrez database revealed that no S1P or LPA gene sequences from Chinese hamster had been deposited. Therefore, semi-redundant oligonucleotides were designed using nucleotide and amino acid sequence alignments for each of the eight genes from human, mouse and rat. These were used to amplify a portion of each of the eight S1P and LPA genes from Chinese hamster genomic DNA. Sequencing of the resultant PCR products generated novel sequence data for these Chinese hamster genes and comparison of partial sequences with human, mouse and rat orthologues demonstrated high levels of identity at both nucleotide and protein levels. The amino acid residue present in TM III, which has been proposed to control S1P and LPA receptor ligand selectivity [Wang *et al.*, 2001], was found

to be present in all sequences analysed (Chinese hamster gene fragments for the S1P₄ and S1P₅ receptors did not cover this region, however it is probable that this residue is also conserved within these sequences). It is therefore unlikely that there would be significant species differences with regard to ligand selectivity among the orthologues for the S1P and LPA receptors. Such differences are sometimes observed and can complicate drug discovery programs due to species-selectivity of ligands and small molecule therapeutics (e.g. chemokine receptors).

Each of the partial hamster receptor sequences analysed shared greatest identity with rodent orthologues and slightly lower identity with the equivalent human sequence. The relative order of identity of Chinese hamster sequences compared to mouse, rat and human orthologues was not consistent with respect to the order of identity with mouse or rat sequences for the eight S1P and LPA receptors, but the rank order for a given receptor was the same at nucleotide and protein levels.

Species-specific oligonucleotides were designed using sequences of the Chinese hamster gene fragments for use in the RT-PCR analysis of Chinese hamster total RNA to indicate which S1P and LPA receptor genes were endogenously expressed in this cell line. This study showed the presence of mRNA for S1P₁, S1P₂, S1P₄ and LPA₁ receptors in CHO-K1 cells, although the result suggested that the S1P₄ transcript was not very abundant since it only yielded a faint product in the RT-PCR experiment. It would have been interesting to continue this study to profile which of these genes were expressed at the protein level, but it was not clear whether the available antibodies to human S1P and LPA receptors would cross-react with hamster protein and generation of hamster-

specific antibodies for these four GPCRs was beyond the scope of this investigation.

The information from this RT-PCR study may be compared with published reports describing expression profiles of the eight S1P and LPA genes in ovarian type tissues. This information is summarised in Table 3.4.

Gene	Previous reports of expression in ovary or related cell lines (method used)	Result from CHO-K1 RT-PCR
S1P ₁	Not detected in CHO-K1 cells using rat S1P ₁ TM domains as probe (Northern blot) ^{1,2}	++
S1P ₂	Expressed in rat ovary (EST expression profiling) ³ Detected in CHO-K1 cells using rat S1P ₂ 5' fragment as probe (Northern blot) ^{1,2}	++
S1P ₃	Not detected in CHO-K1 cells using human S1P ₃ coding sequence as probe (Northern blot) ^{1,2}	-
S1P ₄	Not detected in human ovary (Northern blot) ⁴ Not detected in CHO-K1 cells using human S1P ₄ 3'-coding sequence fragment as probe (Northern blot) ⁵	+
S1P ₅	No data published	-
LPA ₁	Expressed in human ovary (Northern blot) ⁶ Not detected in CHO-K1 cells using human LPA ₁ 3'-UTR as probe (Northern blot) ¹	++
LPA ₂	Not detected in human ovary (Northern blot) ⁶ Not detected in CHO-K1 cells using human LPA ₂ 3'-UTR as probe (Northern blot) ¹	-
LPA ₃	Expressed in human ovary – faint (Northern blot) ⁷	-

Table 3. 4

Summary of S1P and LPA expression data in ovarian tissues and CHO-K1 cells.

Data were taken from published reports and RT-PCR analysis of expression in CHO-K1 cells described in this chapter.

++ strongly positive in RT-PCR; + faintly positive in RT-PCR; - negative in RT-PCR.

References	¹	Okamoto <i>et al.</i> , 1998
	²	Sato <i>et al.</i> , 1999
	³	Glickman <i>et al.</i> , 1999
	⁴	Graeler <i>et al.</i> , 1998
	⁵	Yamazaki <i>et al.</i> , 2000
	⁶	An <i>et al.</i> , 1998
	⁷	Bandoh <i>et al.</i> , 1999

The detection of mRNA for S1P and LPA receptors in CHO-K1 cells did not always correlate with detection of these transcripts in ovarian tissues and probably reflects the heterogeneity of cell types present within ovarian tissues. This investigation confirmed the previous observations, that CHO-K1 cells express S1P₂ but not S1P₃ or LPA₂ [Okamoto *et al.*, 1998], although detection of transcripts for S1P₁ and LPA₁ contradicted this earlier study. This may suggest that the use of species-specific reagents is important in such an investigation, however it should also be noted that the current study used RT-PCR as the analytical tool, which is a more sensitive technique than Northern blotting.

The following chapter describes expression of the HA-S1P₄/pIRES and HA-S1P₄-Gα_{i1}/pIRES constructs in CHO-K1 cells and analysis of the functional responses of the resultant recombinant proteins.

Chapter 4 – Expression and Functional Responses of S1P₄

4.1 Introduction

The lysophospholipid ligand, S1P, is known to elicit myriad cellular responses, including stimulation of calcium flux, alterations in cell morphology and enhanced cell survival (reviewed by Pyne and Pyne, 2000a; Pyne and Pyne, 2000b; Maceyka *et al.*, 2002; Pyne and Pyne, 2002; Spiegel *et al.*, 2002; Hla, 2003; Spiegel and Milstien, 2003). The sensitivity of some of these responses to pertussis toxin was an indicator of the involvement of GPCRs in these processes and the subsequent cloning of high affinity receptors for S1P confirmed a role for this bioactive lipid as a GPCR agonist. Of the six Edg receptors which had been identified when the work described in this chapter was started, three were known to be activated by S1P whilst the two remaining Edg receptors to which ligands had been assigned were activated by LPA [Graeler *et al.*, 1998]. The orphan receptor S1P₄ (then named Edg6) showed greater sequence similarity with the S1P-activated Edg receptors than with those activated by LPA and it therefore seemed possible that S1P might be an agonist for the S1P₄ receptor. Early characterisations of the known S1P receptors showed that all three coupled to G α_i G-proteins and the S1P₂ and S1P₃ receptors could activate multiple G-proteins [Zhang *et al.*, 1999; Kon *et al.*, 1999; Windh *et al.*, 1999] in response to S1P stimulation. Given the common ability of these known S1P receptors to couple to G α_i G-proteins, it was postulated that the S1P₄ receptor would also activate this class of G-proteins.

Activation of a cell-surface GPCR results in the downstream activation of many signalling pathways, which may be conveniently monitored and interpreted with

respect to receptor activation. These include modulation of adenylyl cyclase activity, flux of intracellular calcium and activation of MAPK enzymes. There is a potential advantage, however, in measuring the consequence of receptor activation at the G protein level. Intracellular signalling cascades often involve signal amplification and may be subject to positive and negative influences, which may complicate interpretation of data. The immediate function of GPCR activation is to promote exchange of the guanine nucleotides, GDP and GTP, on the $G\alpha$ G protein and this may be represented as the four stage cycle shown in Figure 4.1.

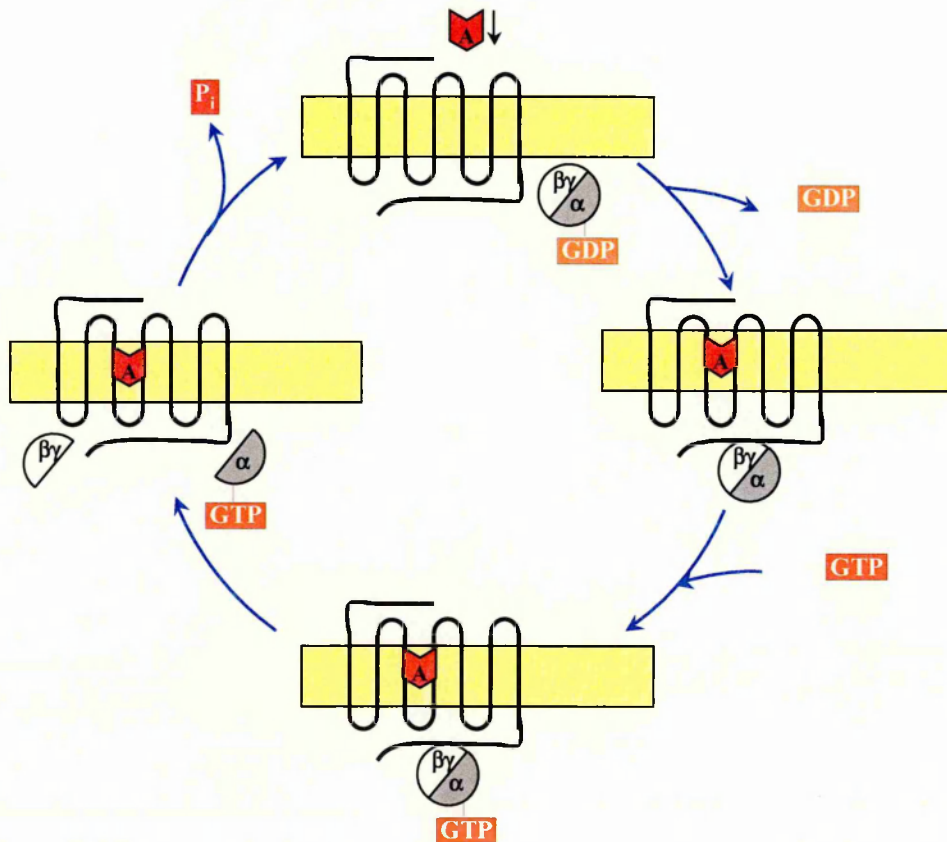


Figure 4. 1
Schematic diagram showing guanine nucleotide exchange cycle on $G\alpha\beta\gamma$ G proteins.

Exchange of guanine nucleotides on $G\alpha\beta\gamma$ G proteins is stimulated by the binding of an agonist (A) to a GPCR located on the cell membrane.

During this cycle, occupation of a GPCR by an agonist causes it to become activated. Guanine nucleotide exchange is initiated by the binding of the activated GPCR to the membrane-associated heterotrimeric G protein. This event stimulates the release of bound GDP from the $G\alpha$ G protein and allows binding of endogenous GTP, which in turn promotes dissociation of the GPCR from the $G\alpha$ -GTP and $G\beta\gamma$ G protein subunits. These $G\alpha$ -GTP and $G\beta\gamma$ G protein subunits then activate effector molecules, such as adenylyl cyclase, phospholipase C and ion channels. The intrinsic GTPase activity of the $G\alpha$ G protein inactivates the $G\alpha$ G protein entity via hydrolysis of the bound GTP back to GDP, with concomitant release of inorganic phosphate. The $G\alpha$ -GDP subunit is then able to reassociate with the $G\beta\gamma$ G protein subunit to reform the GDP-associated heterotrimer, which is ready for another round of activation, thus completing the cycle. The existence of this cycle may be exploited in two ways to measure receptor activation: one possibility is to measure the rate of hydrolysis of $[\gamma^{32}\text{P}]\text{GTP}$ and this is employed in the GTPase assay. Alternatively the binding of a radiolabelled non-hydrolysable analogue of GTP, $[\text{}^{35}\text{S}]\text{GTP}\gamma\text{S}$ may be determined [Windh and Manning, 2002; Milligan, 2003]. These assays are typically performed using crude membranes prepared from cells expressing the receptor of interest.

The focus of the work reported in this chapter was to try to detect direct activation of a G protein in response to stimulation of S1P_4 by S1P using an adapted $[\text{}^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding assay where specific antisera were used to immunoprecipitate $G\alpha_i$ G proteins from detergent extracts prepared from membranes after incubation with S1P, guanine nucleotides and $[\text{}^{35}\text{S}]\text{GTP}\gamma\text{S}$.

This type of GTP γ S/immunoprecipitation (GTP γ S/IP) assay has been successfully used to examine the differential coupling of S1P_{1/2/3} receptors to different G proteins [Windh *et al.*, 1999].

4.2 Results

4.2.1 Transient expression of $\alpha_{2A}AR-G\alpha_{i1}(C^{351}I)$ fusion in HEK-293 cells

HEK-293 cells were transiently transfected to express the $\alpha_{2A}AR-G\alpha_{i1}(C^{351}I)$ fusion protein (detailed in chapter 2, section 3.2), to provide a positive control for use in [^{35}S]GTP γ S binding assays. Approximately 48 hours after transfection, cells were harvested non-enzymatically and membranes prepared, as described in chapter 2, section 4.1. Analysis of the resultant membranes by Western blotting using an anti- $G\alpha_{i1}$ antibody (Figure 4.2) showed the presence of a polypeptide with apparent mass of approximately 40 kDa in both parental and transiently transfected cells and is due to expression of endogenous $G\alpha_{i1}$ G protein. Membranes from transfected cells also contained a polypeptide with apparent M_r of 120 kDa, which reflects expression of the $\alpha_{2A}AR-G\alpha_{i1}(C^{351}I)$ fusion protein. This mass was greater than that predicted on the basis of amino acid sequence and may reflect post-translational modification of the receptor.

4.2.2 Comparison of immunoprecipitation efficiency of different anti-G protein antisera in [^{35}S]GTP γ S binding assay

In order to optimise conditions before work started with $S1P_4$ -transfected membranes, a comparison was made between two antibodies (sc-262 and SG1) for their ability to immunoprecipitate $G\alpha_i$ subunits from HEK-293 membranes transiently expressing $\alpha_{2A}AR-G\alpha_{i1}(C^{351}I)$. A [^{35}S]GTP γ S binding assay was performed, as detailed in chapter 2, section 5.2, using these $\alpha_{2A}AR-G\alpha_{i1}(C^{351}I)$ -expressing membranes, which were incubated with or without the agonist UK14304 (used at a single concentration of 1 μ M).

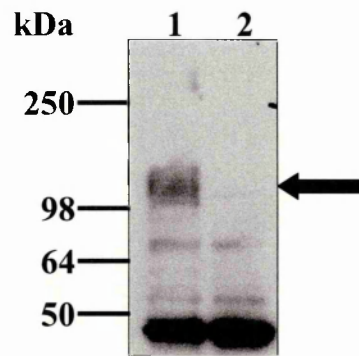


Figure 4. 2

Expression of $\alpha_{2A}AR-G\alpha_{41}(C^{351}I)$ in HEK-293 cells.

Membranes from HEK-293 cells which had been transiently transfected to express the $\alpha_{2A}AR-G\alpha_{41}(C^{351}I)$ fusion protein (lane 1) and untransfected HEK-293 cells (lane 2) were analysed by Western blotting using an anti- $G\alpha_{41}$ antibody. Visualisation of immunoreactive proteins was achieved via chemiluminescence after incubation of the blot with an appropriate HRP-conjugated secondary antibody. The position of the $\alpha_{2A}AR-G\alpha_{41}(C^{351}I)$ fusion is indicated by an arrow.

Both the sc-262 antibody, which cross-reacts with $G\alpha_{41/2/3}$, and the SG1 antiserum, which recognises $G\alpha_{41/2}$ were used at their previously determined maximally effective dilutions of 1 in 100 and 1 in 200, respectively. Whilst an agonist-dependent increase in $[^{35}S]GTP\gamma S$ binding was observed using each antibody, the window of stimulation was significantly greater using the SG1 antiserum (>8-fold compared to 2.5-fold stimulation with sc-262) (Figure 4.3) and may have reflected the ability of SG1 to immunoprecipitate a greater proportion of the $G\alpha_{41/2}$ G protein from the detergent extract. On the basis of these results, the SG1 antiserum was used for the immunoprecipitation step in all subsequent $[^{35}S]GTP\gamma S$ assays.

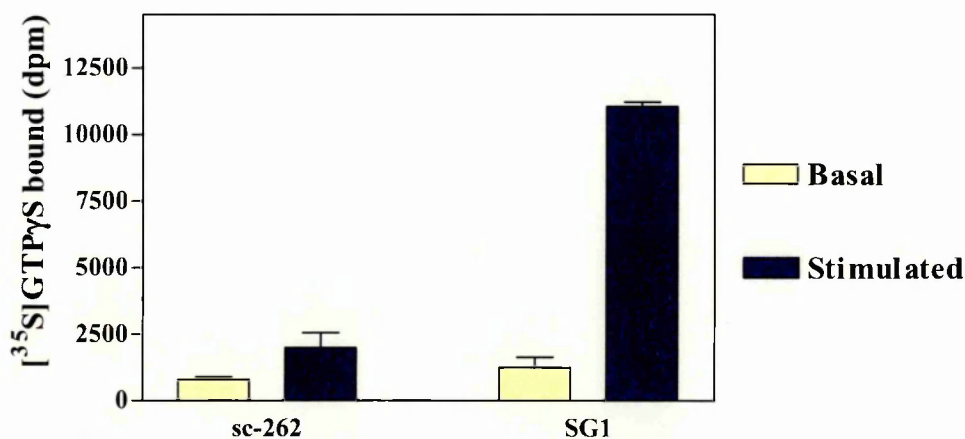


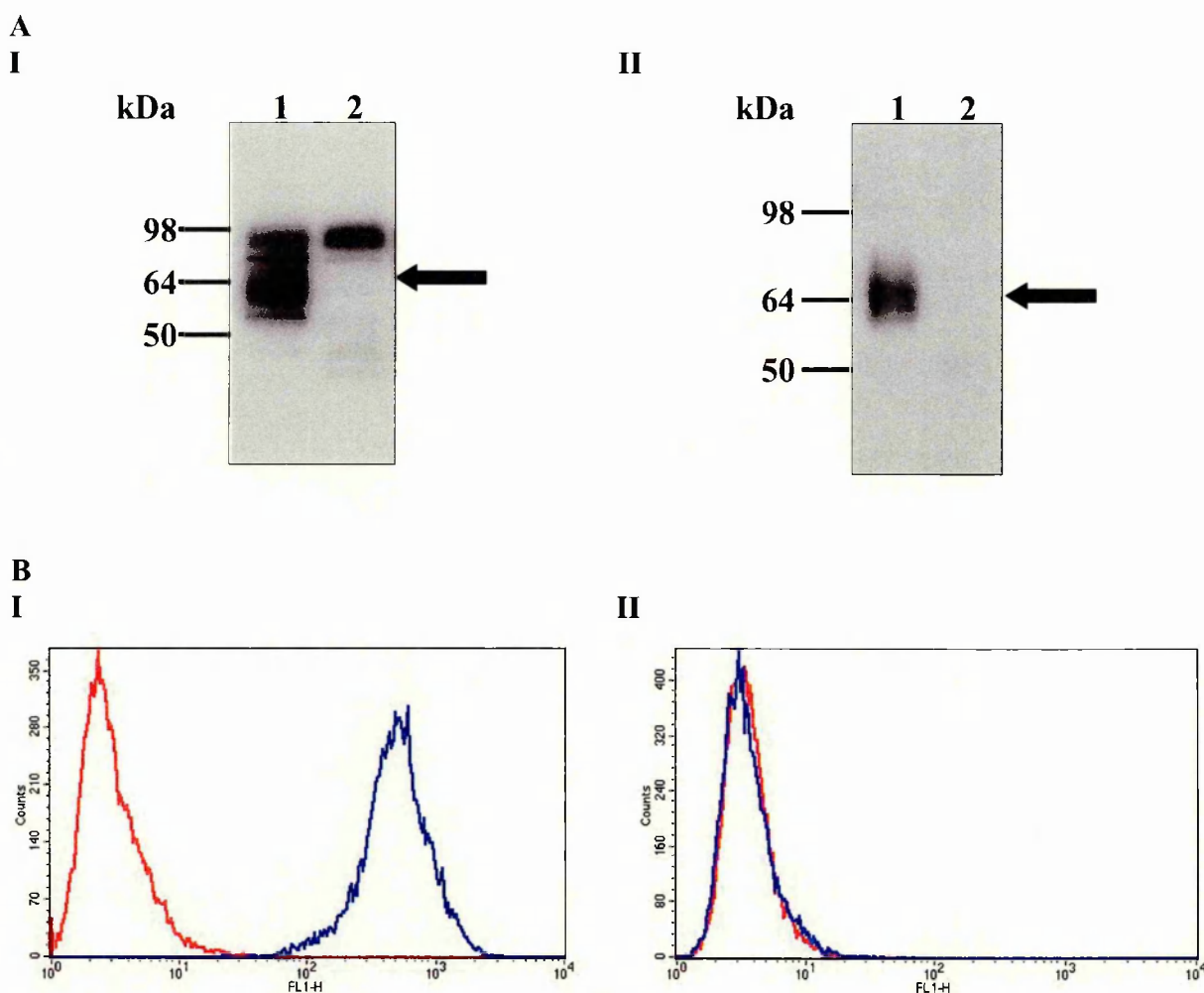
Figure 4. 3

Comparison of different anti-G protein antisera in [^{35}S]GTP γ S binding assay.

Membranes from HEK-293 cells transiently expressing $\alpha_{2A}\text{AR-G}\alpha_{i1}(\text{C}^{351}\text{I})$ were incubated for 10 minutes at 30 °C with [^{35}S]GTP γ S in the absence (basal) or presence (stimulated) of 1 μM UK14304 (an $\alpha_{2A}\text{AR}$ agonist). The membranes were solubilised and immunoprecipitation performed with the sc-262 antibody or SG1 antiserum after preclearance with non-immune serum. Data are from a single experiment and are presented as means of triplicate determinations \pm SEM.

4.2.3 Stable expression of HA-S1P $_4$ in CHO-K1 cells

The plasmid HA-S1P $_4$ -pIRES (described in chapter 3, section 2.1) was stably transfected into CHO-K1 cells, as described in chapter 2, section 3.3. Transfection with the HA-S1P $_4$ construct resulted in the presence of a polypeptide with apparent M_r of 65 kDa, which cross-reacted with anti-HA and anti-S1P $_4$ antibodies (shown in Figure 4.4 (A)) and was consistent with expression of full length HA-tagged S1P $_4$ protein. CHO-K1 membranes (both parental and transfected) expressed a protein of approximately 40 kDa, which was identified using the anti-G α_{i1} antibody and reflected expression of endogenous G α_{i1} G proteins in these cells (not shown). Cell-surface expression of HA-tagged S1P $_4$ was confirmed by FACS analysis using an anti-HA antibody (shown in Figure 4.4 (B)).

**Figure 4. 4****Expression of HA-S1P₄ in CHO-K1 cells.**

A Membranes from CHO-K1 cells stably expressing HA-S1P₄ (lane 1) and untransfected CHO-K1 cells (lane 2) were analysed by Western blotting using anti-S1P₄ (panel I) or anti-HA (panel II) antibodies. Visualisation of immunoreactive proteins was achieved using chemiluminescence after incubation of the blot with appropriate HRP-conjugated secondary antibodies. The position of HA-S1P₄ is indicated by an arrow.

B Cell-surface expressed HA-S1P₄ was detected by FACS analysis using a Fluorescein conjugate of the anti-HA antibody, represented by the blue trace. Cells were also stained with an isotype matched control antibody, denoted by the red trace. Panel I shows the staining obtained using HA-S1P₄-transfected CHO-K1 cells whilst panel II illustrates staining of untransfected CHO-K1 cells. Data are presented as overlay histograms and are representative of at least five independent experiments.

4.2.4 Optimisation of HA-S1P₄ response in [³⁵S]GTPγS binding assay

The concentration of GDP can markedly alter the capacity to monitor specific binding of [³⁵S]GTPγS. To determine the optimal GDP concentration for use in [³⁵S]GTPγS assays with HA-S1P₄, membranes were incubated with or without 10 μM S1P in the presence or absence of varying concentrations of GDP before immunoprecipitation of detergent extracts with the SG1 antiserum (Figure 4.5).

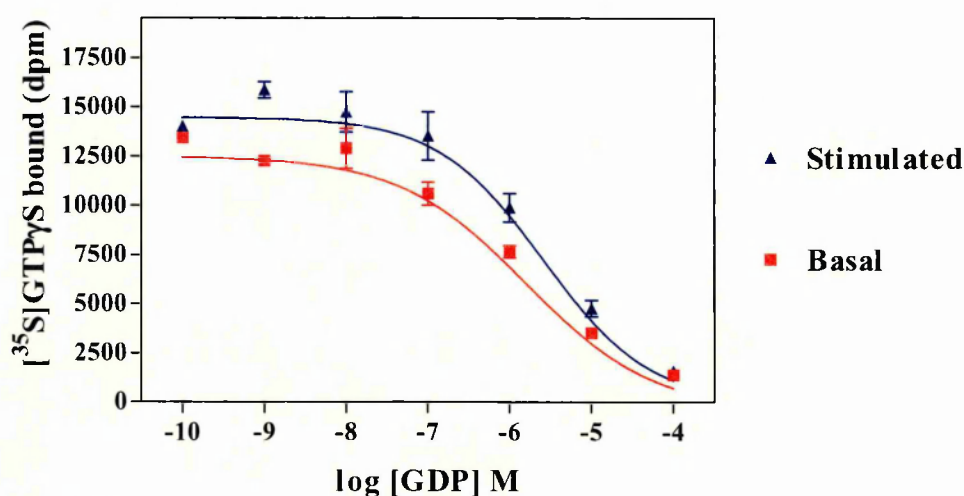


Figure 4. 5

Determination of optimal GDP concentration using HA-S1P₄ in [³⁵S]GTPγS binding assay.

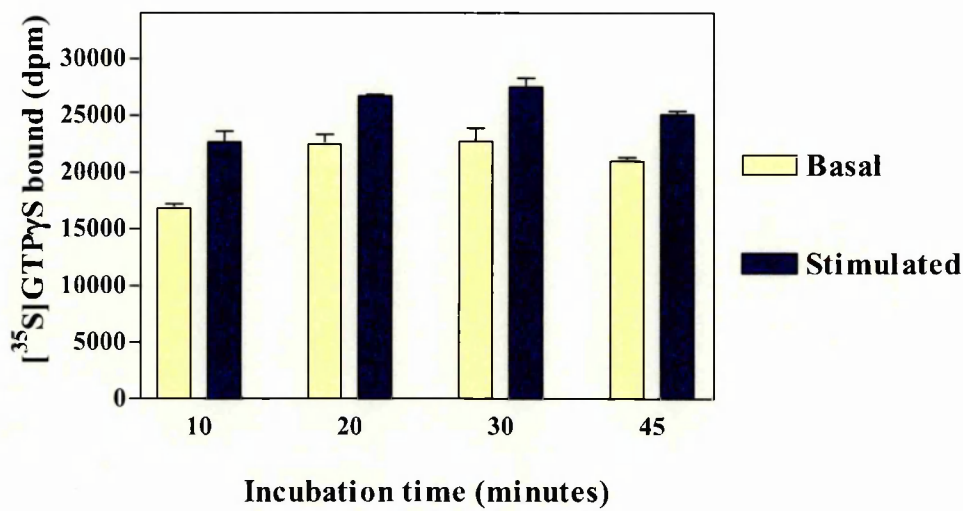
Membranes from CHO-K1 cells stably expressing HA-S1P₄ were incubated for 10 minutes at 30 °C with [³⁵S]GTPγS in the absence (basal) or presence (stimulated) of 10 μM S1P and varying concentrations of GDP. The membranes were then solubilised and SG1 antiserum used to immunoprecipitate Gα_i subunits after preclearance with non-immune serum. Data are presented as means of triplicate determinations ± SEM.

An agonist-dependent increase in [³⁵S]GTPγS binding was observed and was found to be greatest in the presence of 0.1 μM GDP. The level of S1P-mediated stimulation observed at the optimal concentration of GDP was relatively small, so attempts were made to optimise the assay conditions. Time course experiments were performed to determine how the length of incubation could

influence the window of stimulation. Figure 4.6 (A) shows the variation of response with incubation time; the “standard” incubation length of 10 minutes appeared to be preferred to longer incubations, although the general trend of basal and stimulated counts increasing with time suggested that a shorter incubation time could give an improved window of response. The relationship between basal or S1P-stimulated [35 S]GTP γ S binding with time was not linear and appeared to have reached a plateau after incubation for 20 minutes. This may suggest that [35 S]GTP γ S binding was rapid and the linear portion of binding occurred within the first few minutes.

The effect of the permeabilising agent saponin was also examined. Saponin has been reported to increase signal-to-noise ratio without altering pharmacological characteristics of the receptor preparation [Lazareno, 1997] and its effect on the window of stimulation achieved using HA-S1P $_4$ -expressing membranes treated with or without 10 μ M S1P is shown in Figure 4.6 (B). Addition of saponin to assay tubes incubated for 10 minutes was found to increase the level of stimulation over basal from approximately 30 % to greater than 90 % and was therefore chosen to be included in subsequent assays. After a 10 minute incubation, the level of counts in stimulated samples was greater than 10 % of total radioactivity added and so incubation for 5 minutes was deemed preferable, to avoid substrate-depletion effects. This length of incubation gave approximately 2-fold stimulation over basal (shown in Figure 4.6 (B)), with an incorporation of less than 10 % of the radionucleide and was therefore used as the incubation time for subsequent assays of HA-S1P $_4$.

A



B

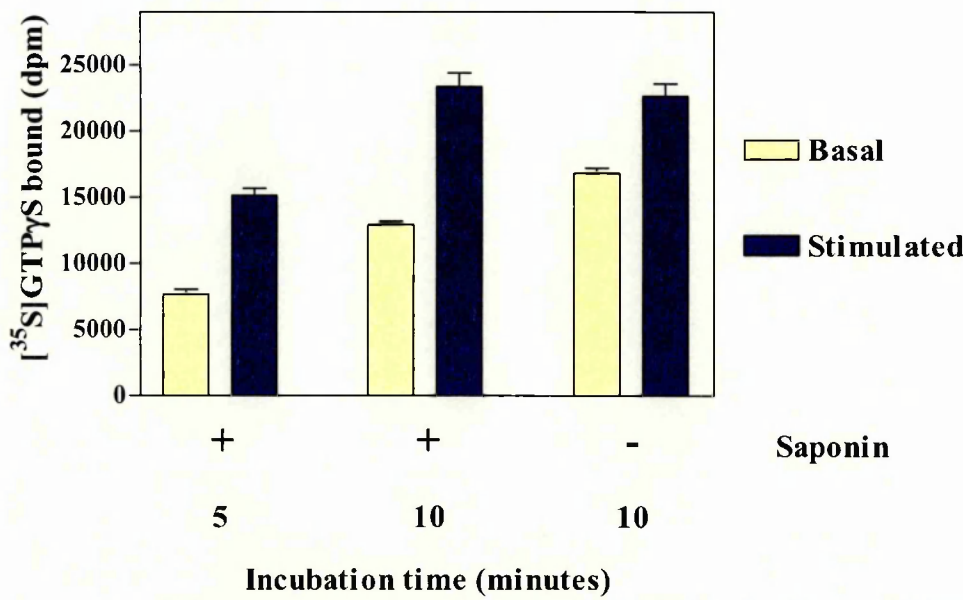


Figure 4. 6
Optimisation of response of HA-S1P₄ in [³⁵S]GTPγS binding assay.
Membranes from CHO-K1 cells stably expressing HA-S1P₄ were assayed for [³⁵S]GTPγS binding in the absence (basal) or presence (stimulated) of 10 μM S1P. The assay was incubated at 30 °C for the times shown (A) and where indicated, saponin was included in the assay mix (B). The membranes were then solubilised and SG1 antiserum used to immunoprecipitate Gα_i subunits after preclearance with non-immune serum. Data are the mean of three determinations ± SEM from a single experiment and are representative of two independent experiments.

4.2.5 Constitutive activity of HA-S1P₄ in [³⁵S]GTPγS binding assay

In comparison with membranes expressing α_{2A}AR-Gα_{i1}, the level of basal [³⁵S]GTPγS binding observed using HA-S1P₄-expressing membranes during the initial optimisation experiments was noted as being markedly higher. Although this could have been due to the difference in host system (α_{2A}AR-Gα_{i1} was expressed in HEK-293 cells whilst HA-S1P₄ was expressed CHO-K1 cells), this observation coupled with the relatively small effects of S1P suggested that HA-S1P₄ might be constitutively active. This was examined further: [³⁵S]GTPγS binding was measured using HA-S1P₄-transfected, or untransfected, CHO-K1 membranes treated with or without 10 μM S1P, or with a vehicle (0.1 % fatty acid free BSA) control. Expression of HA-S1P₄ in CHO-K1 membranes resulted in a significant increase in the level of basal [³⁵S]GTPγS binding (Figure 4.7). This agonist-independent activity represented constitutive activity of the S1P₄ receptor and was also observed with a second clone expressing the same construct (data not shown). Treatment with S1P further increased the extent of [³⁵S]GTPγS binding in HA-S1P₄-expressing membranes and was shown to be S1P₄-dependent since no such increase was observed using untransfected membranes.

4.2.6 Pertussis toxin-sensitivity of HA-S1P₄ dose-response to S1P in [³⁵S]GTPγS binding assay

The previous observations that suggested HA-S1P₄ was constitutively active were examined in detail during the course of EC₅₀ determinations for the ligand S1P. S1P-mediated increases in [³⁵S]GTPγS binding were concentration-

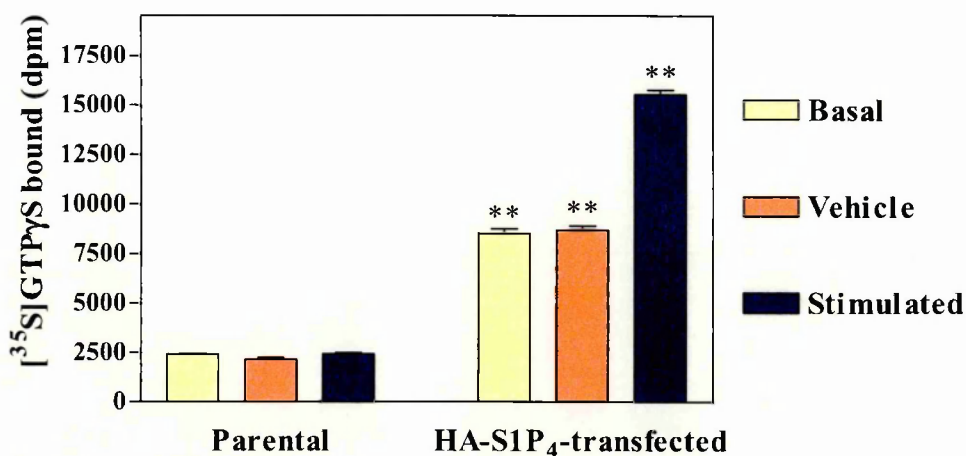


Figure 4. 7

Constitutive activity of HA-S1P₄ after expression in CHO-K1 cells.

Membranes from parental or HA-S1P₄-transfected CHO-K1 cells were incubated for 5 minutes at 30 °C with [³⁵S]GTPγS in the absence (basal) or presence (stimulated) of 10 μM S1P, or 0.1 % fatty acid free BSA vehicle control (vehicle). G protein α subunits were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data are the mean of three determinations ± SEM from a single experiment, and are representative of three such experiments performed. Statistical significance from the basal response of parental cells is denoted by ** (P < 0.01).

dependent (Figure 4.8). The mean EC₅₀ obtained for activation of HA-S1P₄ by S1P was 565 ± 245 nM (n=4). Both S1P-induced and agonist-independent [³⁵S]GTPγS binding were shown to be sensitive to ADP-ribosylation by pertussis toxin. Pre-treatment of cells with 100 ng/mL pertussis toxin for 24 hours prior to harvesting resulted in [³⁵S]GTPγS binding levels that were unaffected by the presence of S1P and were similar to those seen using parental CHO-K1 membranes (Figure 4.7). A second CHO-K1 clone expressing HA-S1P₄ gave similar results (data not shown), with an EC₅₀ for activation by S1P of 320 nM (n=1), significant constitutive activity and complete sensitivity of basal and agonist-induced [³⁵S]GTPγS binding to ADP-ribosylation by pertussis toxin.

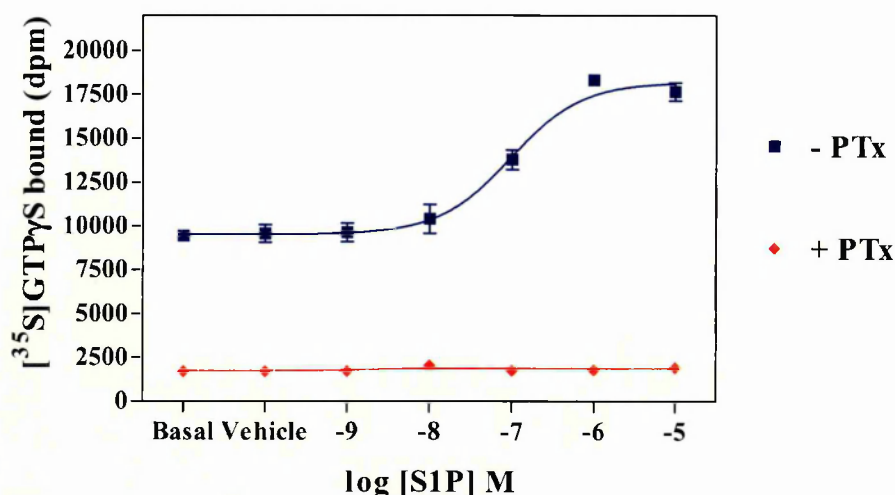


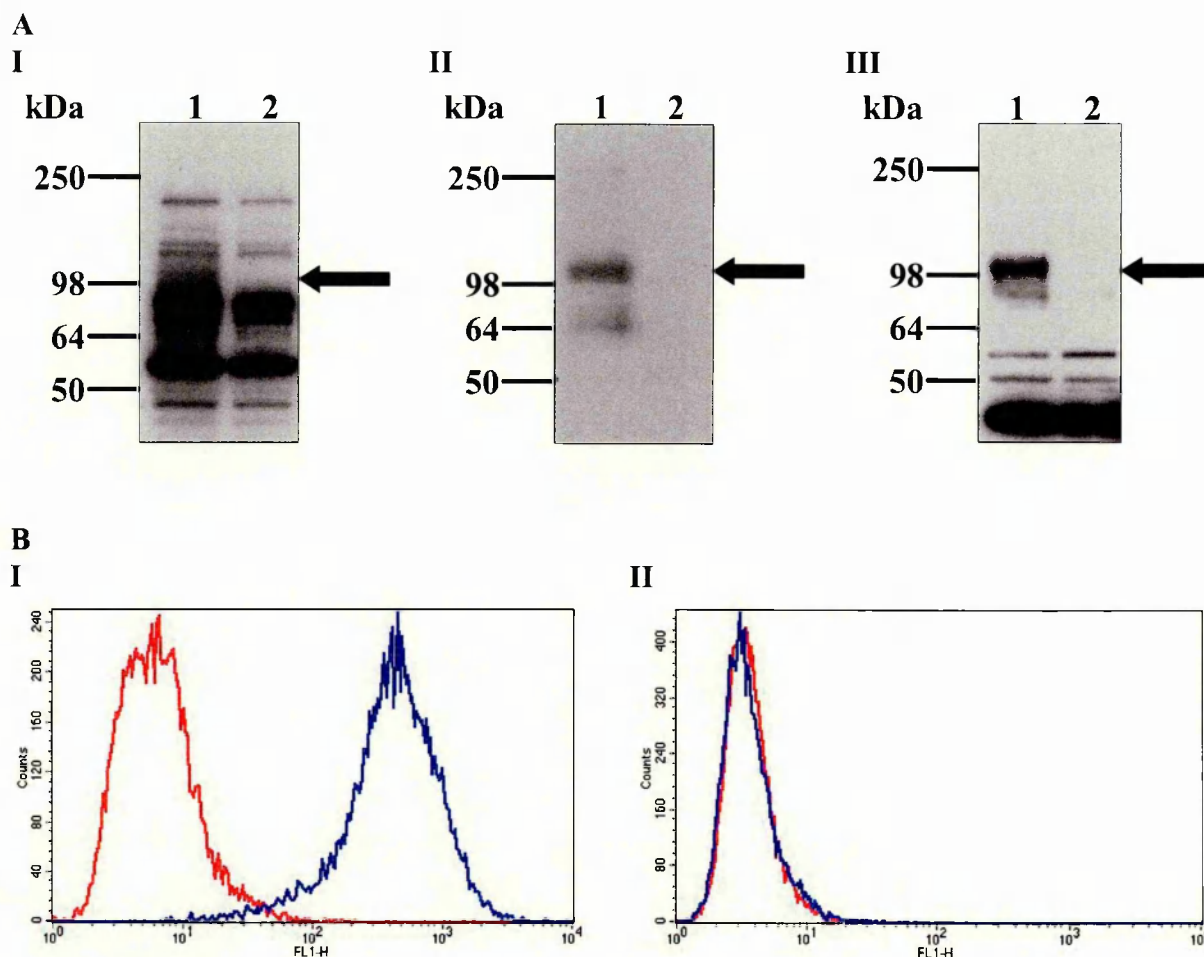
Figure 4. 8

Concentration dependent stimulation of HA-S1P₄-mediated [³⁵S]GTPγS binding by S1P.

Membranes from CHO-K1 cells transfected with HA-S1P₄ which had been cultured in the absence (- PTx) or presence (+ PTx) of 100 ng/mL pertussis toxin for 24 hours prior to harvest were stimulated with varying concentrations of S1P for 5 minutes at 30 °C in the [³⁵S]GTPγS/IP binding assay. Gα_i G proteins were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data are the mean of three determinations ± SEM from a single experiment, and are representative of four such experiments performed.

4.2.7 Stable expression of HA-S1P₄-Gα_{i1}(C³⁵¹I) fusion in CHO-K1 cells

CHO-K1 cells were stably transfected with the plasmid HA-S1P₄-Gα_{i1}(C³⁵¹I) (described in chapter 3, section 2.2). Membranes from cells expressing HA-S1P₄-Gα_{i1}(C³⁵¹I) contained a polypeptide with an apparent M_r of 110 kDa which reacted with the anti-HA, anti-S1P₄ and anti-Gα_{i1} antibodies, (Figure 4.9 (A)). This was indicative of expression of the HA-tagged S1P₄-Gα_{i1}(C³⁵¹I) fusion protein. As had been observed with the unfused HA-S1P₄-transfected membranes, the apparent mass of the HA-S1P₄-Gα_{i1}(C³⁵¹I) was greater than that predicted on the basis of amino acid sequence and suggested the presence of post-translational modifications. FACS analysis using the anti-HA antibody was used to confirm the presence of cell-surface fusion protein (Figure 4.9 (B)).

**Figure 4. 9****Expression of HA-S1P₄-Gα_{i1}(C³⁵¹I) in CHO-K1 cells.**

A Membranes from CHO-K1 cells stably expressing HA-S1P₄-Gα_{i1}(C³⁵¹I) (lane 1) and untransfected CHO-K1 cells (lane 2) were analysed by Western blotting using anti-S1P₄ (panel I), anti-HA (panel II) or anti-Gα_{i1} (panel III) antibodies. Visualisation of immunoreactive proteins was achieved using chemiluminescence after incubation of the blot with appropriate HRP-conjugated secondary antibodies. The position of HA-S1P₄-Gα_{i1}(C³⁵¹I) is indicated by an arrow.

B Cell-surface expressed HA-S1P₄-Gα_{i1}(C³⁵¹I) was detected by FACS analysis using a Fluorescein conjugate of the anti-HA antibody, represented by the blue trace. Cells were also stained with an isotype matched control antibody, denoted by the red trace. Panel I shows the staining obtained using HA-S1P₄-Gα_{i1}(C³⁵¹I)-transfected CHO-K1 cells whilst panel II illustrates staining of untransfected CHO-K1 cells. Data are presented as overlay histograms and are representative of at least five independent experiments.

4.2.8 Optimisation of HA-S1P₄-Gα_{i1}(C³⁵¹I) response in [³⁵S]GTPγS binding assay

A similar approach to that used with the HA-S1P₄-expressing membranes (detailed in this chapter, section 2.4) was applied to optimise the response of HA-S1P₄-Gα_{i1}(C³⁵¹I) in the [³⁵S]GTPγS binding assay. Figure 4.10 shows the effect of GDP concentration on the ability to detect agonist-mediated increases in [³⁵S]GTPγS binding. Similar to the result obtained with the unfused HA-S1P₄ construct, the optimal concentration of GDP was approximately 0.1 μM. Time course experiments were performed to determine the optimal incubation time for this receptor construct. Saponin was included in these incubations as it had successfully increased the window of stimulation observed with the unfused HA-S1P₄ receptor (described in this chapter, section 2.4).

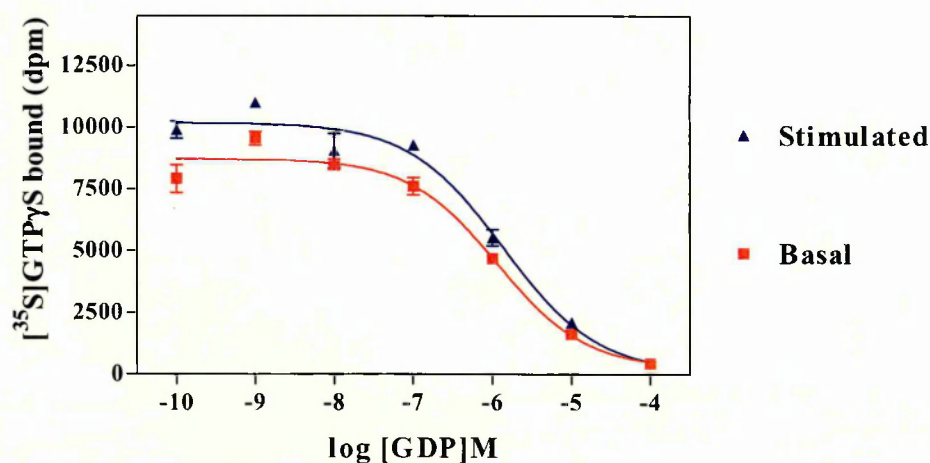


Figure 4. 10

Determination of optimal GDP concentration using HA-S1P₄-Gα_{i1}(C³⁵¹I) in [³⁵S]GTPγS binding assay.

Membranes from CHO-K1 cells stably expressing HA-S1P₄-Gα_{i1}(C³⁵¹I) were incubated for 10 minutes at 30 °C with [³⁵S]GTPγS in the absence (basal) or presence (stimulated) of 10 μM S1P and varying concentrations of GDP. The membranes were then solubilised and SG1 antiserum used to immunoprecipitate Gα_i subunits after preclearance with non-immune serum. Data are presented as means of triplicate determinations ± SEM.

Figure 4.11 shows the effect of incubation time on the stimulation window. In contrast to the unfused HA-S1P₄ construct, which gave an optimal level of stimulation above basal after 5-10 minutes, the optimal incubation time for the HA-S1P₄-Gα_{i1}(C³⁵¹I) fusion was 30 minutes and was used in subsequent experiments. Similar to observations with the unfused HA-S1P₄ receptor, basal and agonist-stimulated [³⁵S]GTPγS binding did not show a linear relationship with time which suggested that the linear portion of [³⁵S]GTPγS binding occurred in the first few minutes.

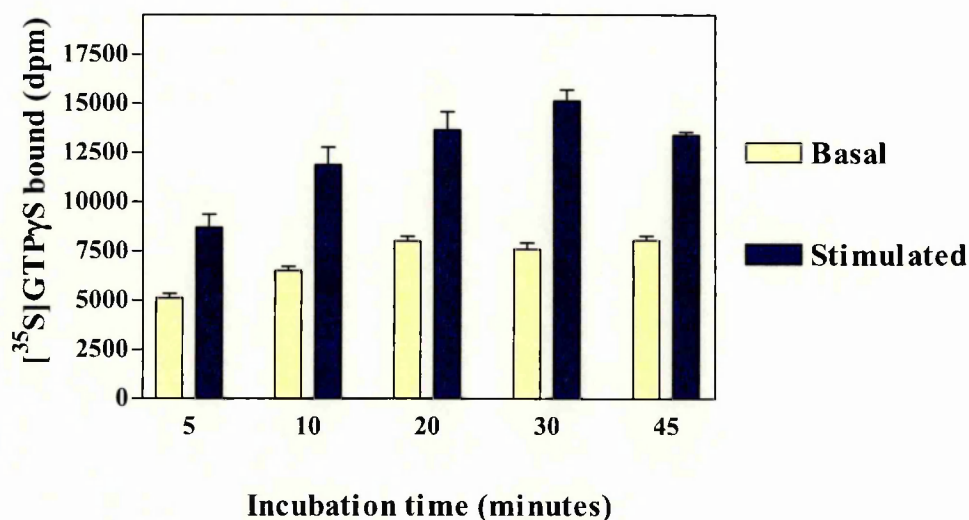


Figure 4. 11

Optimisation of response of HA-S1P₄-Gα_{i1}(C³⁵¹I) in [³⁵S]GTPγS binding assay.

Membranes from CHO-K1 cells stably expressing HA-S1P₄Gα_{i1}(C³⁵¹I) were assayed for [³⁵S]GTPγS binding in the absence (basal) or presence (stimulated) of 10 μM S1P and incubated at 30 °C for varying lengths of time. Post assay, membranes were solubilised and Gα_i subunits immunoprecipitated using SG1 antiserum after preclearance with non-immune serum. Data shown are the mean of three determinations ± SEM from a single experiment and are representative of two independent experiments.

4.2.9 Constitutive activity of HA-S1P₄-Gα_{i1}(C³⁵¹I) in [³⁵S]GTPγS binding assay

Measurement of [³⁵S]GTPγS binding using HA-S1P₄-Gα_{i1}(C³⁵¹I)-transfected or untransfected CHO-K1 membranes was made after treatment with or without 10 μM S1P, or with a vehicle (0.1 % fatty acid free BSA) control. Expression of HA-S1P₄-Gα_{i1}(C³⁵¹I) in CHO-K1 cells resulted in higher levels of basal [³⁵S]GTPγS binding than that observed in untransfected CHO-K1 cells and treatment with S1P further increased [³⁵S]GTPγS binding in HA-S1P₄-Gα_{i1}(C³⁵¹I)-expressing membranes in a receptor-dependent manner (Figure 4.12). This was similar to observations made with the unfused HA-S1P₄ receptor (this chapter, section 2.5).

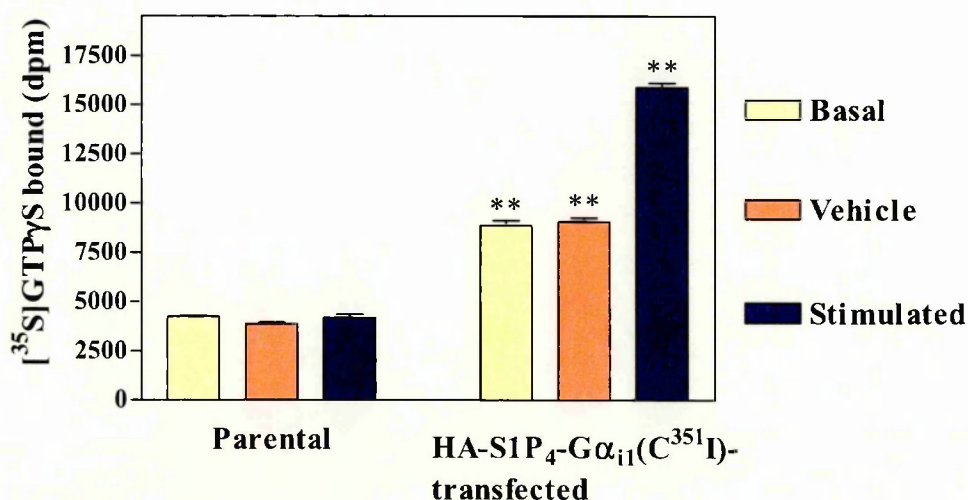


Figure 4. 12

Constitutive activity of HA-S1P₄-Gα_{i1}(C³⁵¹I) expressed in CHO-K1 cells.

Membranes from parental or HA-S1P₄-Gα_{i1}(C³⁵¹I)-transfected CHO-K1 cells were incubated with [³⁵S]GTPγS in the absence (basal) or presence (stimulated) of 10 μM S1P, or 0.1 % fatty acid free BSA vehicle control (vehicle) for 30 minutes at 30 °C. G protein α subunits were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data are the mean of three determinations ± SEM from a single experiment, and are representative of three such experiments performed. Statistical significance from the basal response of parental cells is denoted by ** (P < 0.01).

4.2.10 Pertussis toxin-sensitivity of HA-S1P₄-G α_{i1} (C³⁵¹I) dose-response to S1P in [³⁵S]GTP γ S binding assay

The potency of the ligand S1P as an agonist of the HA-S1P₄-G α_{i1} (C³⁵¹I) fusion construct was determined in the [³⁵S]GTP γ S binding assay (Figure 4.13). The EC₅₀ for S1P activation was 355 nM \pm 155 nM (n=3). In contrast to the pertussis toxin-sensitivity seen with HA-S1P₄ membranes, HA-S1P₄-G α_{i1} (C³⁵¹I) membranes treated with pertussis toxin for 24 hours prior to harvest were able to exhibit increased [³⁵S]GTP γ S binding after S1P stimulation, with an EC₅₀ of 805 nM \pm 190 nM (n=3) which reflected activation of the fused, pertussis toxin-insensitive G α_{i1} G protein. This construct also appeared to be constitutively active because basal counts obtained, even after pertussis toxin treatment, were appreciably higher than those obtained with untransfected CHO-K1 membranes incubated for the same length of time under the same assay conditions (shown in Figure 4.12). There was a marked reduction in basal [³⁵S]GTP γ S binding, however, following pertussis toxin treatment. This may reflect the ability of HA-S1P₄-G α_{i1} (C³⁵¹I) to interact constitutively with endogenously expressed G_i G proteins. Pretreatment with pertussis toxin also produced a small decrease in the extent of the signal produced by S1P. This also suggested a degree of coupling of the HA-S1P₄-G α_{i1} (C³⁵¹I) fusion to endogenous pertussis toxin-sensitive G proteins. However, the majority of the signal from the fusion protein was clearly mediated via the fused, pertussis toxin-insensitive G protein. A second clone expressing the HA-S1P₄-G α_{i1} (C³⁵¹I) protein was also tested (data not shown). This clone expressed very low levels of HA-S1P₄-G α_{i1} (C³⁵¹I) and whilst EC₅₀ values for activation by S1P were somewhat higher than for the original clone tested, the overall profile of response was similar: constitutive activity was

evident even after pertussis toxin treatment and S1P was able to stimulate activation of the receptor both before and after pretreatment of cells with pertussis toxin.

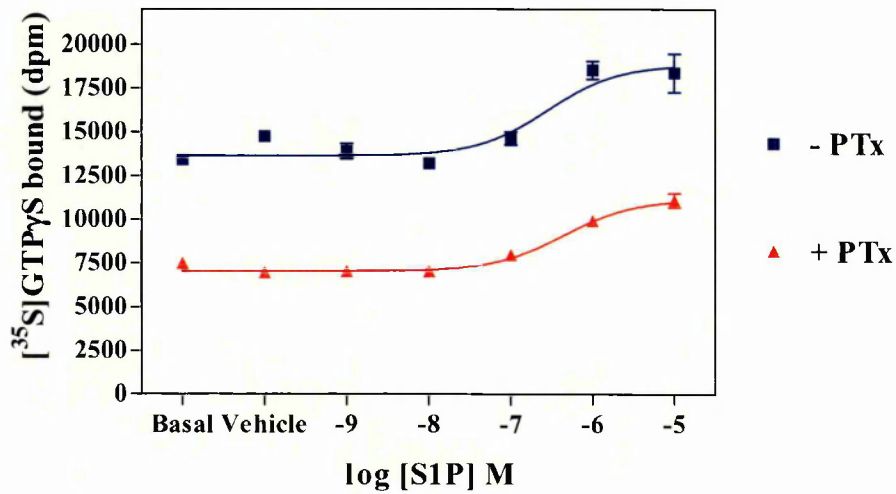


Figure 4. 13

Concentration dependent stimulation of HA-S1P₄-Gα_{i1}(C³⁵¹I)-mediated [³⁵S]GTPγS binding by S1P.

Membranes from CHO-K1 cells transfected with HA-S1P₄-Gα_{i1}(C³⁵¹I) which had been cultured in the absence (- PTx) or presence (+ PTx) of 100 ng/mL pertussis toxin for 24 hours prior to harvest were stimulated with varying concentrations of S1P for 30 minutes at 30 °C in the [³⁵S]GTPγS/IP binding assay. Gα_i G proteins were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data are the mean of three determinations ± SEM from a single experiment, and are representative of three such experiments performed.

4.3 Discussion

S1P₄ was cloned as the orphan GCPR named Edg6 [Graeler *et al.*, 1998]. Sequence similarity with other Edg receptors suggested the lysophospholipid S1P might be an agonist of this receptor, and that it may couple to G α_i G proteins. This hypothesis was tested in this chapter.

Initially, HA-S1P₄ was expressed in RH7777 cells, which have been reported to be unresponsive to S1P and LPA [Zhang *et al.*, 1999]. Preliminary attempts had been made to measure S1P-induced activation of the S1P₄ receptor expressed in this cell line using conventional [³⁵S]GTP γ S binding, inhibition of cAMP accumulation and calcium flux assays. Unfortunately, it was not possible to detect activation of this receptor in these assays. The receptor was then expressed in CHO-K1 cells and the modified [³⁵S]GTP γ S/IP binding assay used to try to measure activation of S1P₄ at the level of receptor-G protein interaction.

The concentration of GDP used in this assay was optimised since GDP affects the magnitude of agonist stimulated [³⁵S]GTP γ S binding [Windh and Manning, 2002]. High GDP concentrations suppress basal activity whilst at low GDP concentrations, basal activity is unrestricted so the ability of an agonist to further stimulate [³⁵S]GTP γ S binding is limited. The optimal concentration of GDP for the detection of S1P-stimulated [³⁵S]GTP γ S binding for the S1P₄ receptor was 0.1 μ M. The time of incubation was optimised such that the window of stimulation was maximised. The non-linear profile of the timecourses suggested that [³⁵S]GTP γ S binding plateaued rapidly and was not unexpected, since a similar profile was reported for the 5-HT_{1A} receptor [Windh and Manning, 2002]. Further optimisation of the signal was obtained via the addition of the

permeabilising agent saponin. In this study, saponin was used at a 1:1 ratio relative to the amount of membranes, however it may have been beneficial to test other ratios. A study of the SLC-1 receptor (somatostatin-like receptor 1) by Audinot and *et al.*, [2001] investigated the effect of different saponin ratios on ligand-induced [35 S]GTP γ S binding for this receptor. Saponin was shown to produce a bell-shaped curve and the peak stimulation corresponded to a ratio of approximately 1:2.5 (saponin:SLC-1 membranes).

Whilst this work was in progress, two independent groups reported the ability of S1P to bind to S1P $_4$ and stimulate ERK phosphorylation [Van Brocklyn *et al.*, 2000] and calcium flux [Yamazaki *et al.*, 2000]. However, this receptor was reported to be non-functional in a study using a conventional GTP γ S assay to compare the activation of S1P $_{1/2/3}$ with S1P $_5$, expressed in HEK-293 cells, by various analogues of S1P [Im *et al.*, 2001a]. Therefore the results described in this chapter were the first demonstration of S1P $_4$ activation measured at the G protein level. Subsequently, two groups have shown activation of S1P $_4$ using a conventional [35 S]GTP γ S assay [Brinkmann *et al.*, 2002; Mandala *et al.*, 2002] and the data presented in this chapter concurs with these reports.

In this chapter, it has been shown that 1) S1P $_4$ couples to G α_i G proteins; 2) S1P $_4$ is strongly constitutively active when expressed in CHO-K1 cells and 3) S1P $_4$ can be further activated by treatment with S1P. These results support published observations that S1P $_4$ is activated by S1P [Van Brocklyn *et al.*, 2000; Yamazaki *et al.*, 2000] but in addition are the first to show the extent of constitutive activity displayed by S1P $_4$. It may be that previous difficulties in observing S1P activation of S1P $_4$ reflect this high level of constitutive activity. The increase in

[³⁵S]GTPγS binding in S1P₄-transfected CHO-K1 membranes when compared to untransfected membranes is strongly indicative of a high level of constitutive activity of the receptor and warrants further investigation. Additionally, the S1P₄ receptor was seen to exhibit significant levels of basal activity in a high affinity GTPase assay (data not shown). The ability of S1P₄ to couple to Gα_i G proteins is a feature shared by all of the S1P-activated Edg receptors. In common with S1P_{2/3/5}, S1P₄ couples to multiple G proteins and has been reported to activate Gα_i and Gα_{12/13} [Van Brocklyn *et al.*, 2000; Yamazaki *et al.*, 2000; Graeler *et al.*, 2003].

The use of fusion proteins constructed between a GPCR and the α subunit of a G protein has become widespread and offers several advantages over systems where non-fused receptors are studied (reviewed by Seifert *et al.*, 1999; Milligan, 2000; Wurch and Pauwels, 2001). Construction of a GPCR-Gα fusion using a mutated Gα G protein that is insensitive to ADP-ribosylation by pertussis toxin allowed isolation of the response from the fused receptor [Milligan, 2000]. In this study, a pertussis toxin-insensitive HA-S1P₄-Gα_{i1}(C³⁵¹I) fusion protein was used, enabling the elimination of signalling from endogenously expressed S1P receptors by treatment with pertussis toxin. The ability of the tethered G protein to transduce the activation of S1P₄ was demonstrated using membranes prepared from pertussis toxin-treated cells, where S1P was shown to promote a dose-dependent increase in [³⁵S]GTPγS binding. The HA-S1P₄-Gα_{i1}(C³⁵¹I) fusion protein also exhibited constitutive activity.

The lysophospholipid, S1P has been identified as the high affinity ligand for several members of the Edg receptor family and these S1P receptors bind S1P

with dissociation constants in the range 2 nM for S1P₅ to approximately 25 nM for S1P_{2/3} [Lee *et al.*, 1998; Kon *et al.*, 1999; Im *et al.*, 2000a]. In a recent parallel study [Im *et al.*, 2001a] a conventional GTPγS binding assay was used to compare the efficacy of various agonists, including S1P, to activate S1P_{1/2/3} and S1P₅, using membranes prepared from HEK-293 transfected cells. Values for the EC₅₀ of S1P-induced GTPγS binding were in the range 0.9 – 2.9 nM for S1P_{1/2/3} and 44 nM for S1P₅; these data may be compared with EC₅₀ values obtained for activation of the S1P₄ constructs described here: 565 nM for HA-S1P₄ and 355 - 805 nM for the HA-S1P₄-Gα₁₁(C³⁵¹I) fusion protein. Statistical analysis indicated that these EC₅₀ values were not significantly different. This probably reflects the standard errors associated with these values and is a result of each S1P dose-response experiment having been set up using membranes prepared from separate cell passages (which expressed different levels of cell-surface S1P₄). S1P has previously been shown to bind to the S1P₄ receptor, albeit with a lower affinity than that with which it binds other S1P receptors. Dissociation constants for S1P binding to S1P₄ expressed in mammalian cells fall in the range 20 nM – 95 nM [Van Brocklyn *et al.*, 2000; Yamazaki *et al.*, 2000; Mandala *et al.*, 2002]. Therefore, whilst S1P does bind to, and activate S1P₄, it does so with lower affinity and potency than it does at the other S1P receptors, which may suggest that it is not the endogenous ligand for the S1P₄ receptor. Phylogenetic analysis of the Edg receptors (shown in chapter 1, Figure 1.6) shows that whilst the other S1P receptors form a discrete cluster, S1P₄ is on the edge of this cluster and could represent the first member of a new subset of Edg receptors, which are activated by a lipid that is structurally distinct from S1P and LPA. A recent survey of GPCRs in the human genome [Vassilatis *et al.*,

2003] predicted that the Edg receptor family will not extend past the eight members already cloned. It should be noted however, that a related family of orphan GPCRs (GPR3, 6 and 12) were recently identified as S1P receptors, indicating that there are certainly other GPCRs which respond to S1P and related lipids [Uhlenbrock *et al.*, 2002].

In the following chapter, further investigations into the constitutive activity displayed by S1P₄ are described.

Chapter 5 – Use of an Inducible Expression System to Examine the Constitutive Activity of S1P₄

5.1 Introduction

In chapter 4 it was shown that the S1P₄ receptor is constitutively active and whilst this may have been a consequence of the high levels of recombinant receptor present in membranes used for these investigations, it could also reflect an inherent property of S1P₄. This chapter describes work carried out to further characterise the ability of S1P₄ to signal in the absence of ligand stimulation.

Constitutive activity is defined as ligand-independent activity and results in G protein activation and second messenger production in the absence of stimulation by an agonist. The phenomenon of GPCR constitutive activity has been widely reviewed [Lefkowitz *et al.*, 1993; Leurs *et al.*, 1998; de Ligt *et al.*, 2000] although it is not yet clear whether this property is physiologically relevant. Ligand-independent activation may be observed for almost any GPCR, although the introduction of a constitutively activating mutation (CAM) is often required to induce significant levels of agonist-independent activity (reviewed by Parnot *et al.*, [2002]).

The finding that certain receptors exhibit constitutive activity has led to a modification of traditional receptor theory [Samama *et al.*, 1993]. It is now believed that receptors can exist in at least two conformations, an inactive (R) and an active (R*) state and the equilibrium between these two conformations strongly favours the inactive receptor state. In some native receptors, and in those which have been modified by the introduction of a constitutively activating mutation, this equilibrium appears to be shifted so that there is a sufficient

number of receptors in the active state to initiate signalling, in the absence of agonist stimulation.

Constitutive activity is sensitive to the actions of inverse agonists. These ligands were previously defined as antagonists, since they were known to bind to, but not activate GPCRs. The discovery that many of these as “antagonists” were able to decrease agonist-independent activity of constitutively active receptors, and therefore possessed negative efficacy, led to their reclassification as inverse agonists. Inverse agonists have now been characterised for many receptor families and are valuable pharmacological tools that allow definitive demonstration of GPCR constitutive activity. At the time of these investigations however, no such compounds had been identified for the S1P receptor family.

Studies into the effect of expression level on receptor functionality may be undertaken as a way of characterising constitutive activity in the absence of such pharmacological tools. One of the more simple methods is to isolate a number of clonal cell lines which express a range of receptor densities and use these cell line in subsequent functional assays. This method utilises the positive correlation which exists between receptor density and agonist-independent activity. A potential drawback with such an approach is that it may not be possible to isolate clones which express significantly different levels of receptor, and by definition, comparisons would be made between different cell lines.

An attractive alternative is to employ an expression system that permits control over the level of protein expression via incubation of the cells with an agent which drives transcription of the gene of interest (reviewed by Clackson [1997]). This technique has the advantage of permitting examination into the effect of receptor expression level using the same clonal population of cells. The key

prerequisites for such a system are low levels of uninduced (background) expression and a specific induction agent which induces a large range of expression levels, in a dose-dependent manner. This is generally achieved using a heterologous transcription factor which binds a DNA sequence that is not recognised by endogenous transcription factors, and a small molecule which can modulate the activity of the transcription factor. There are two such commercially available systems: the tetracycline- and ecdysone-inducible mammalian expression systems; the latter of these was chosen for use in studies with the S1P₄ receptor.

The edysone-inducible expression system was first used to regulate expression of genes in HEK-293 cells [Christopherson *et al.*, 1992] and is classified as an “allosteric on-switch” system [Clackson, 1997] i.e. transcription is stimulated by exposure to the induction agent. It is based on the *Drosophila* moulting induction system, which has been modified for use in mammalian systems and uses analogues of the ecdysone steroid hormone to potently activate expression of the gene of interest via a heterodimeric nuclear receptor composed of a modified form of the ecdysone receptor and the retinoid X receptor. Figure 5.1 shows a schematic diagram of this system. The ecdysone-inducible system has been used for the inducible expression of numerous recombinant proteins, including the somatostatin SST₂ and dopamine D2_{long} GPCRs [Choi *et al.*, 2000; Cole *et al.*, 2001].

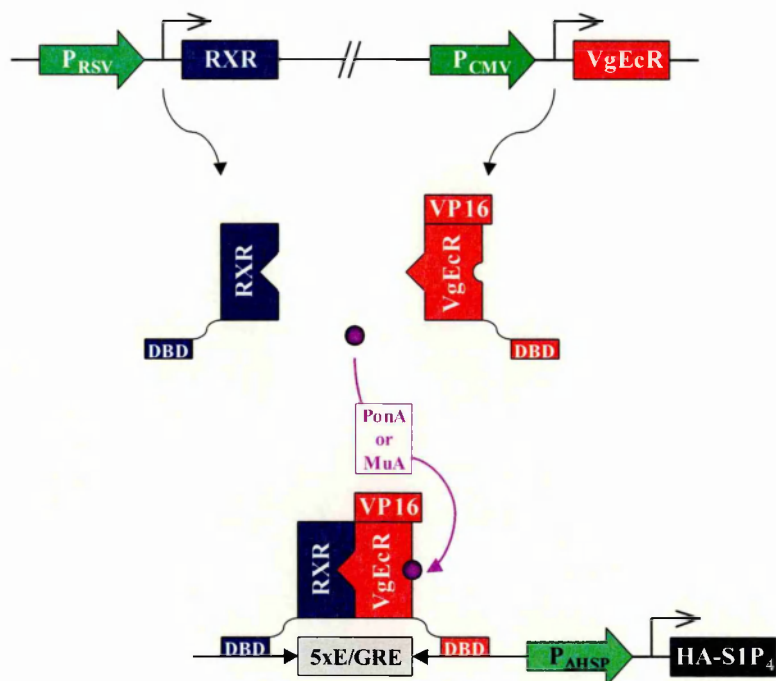
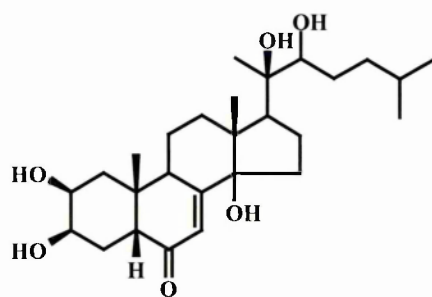


Figure 5. 1

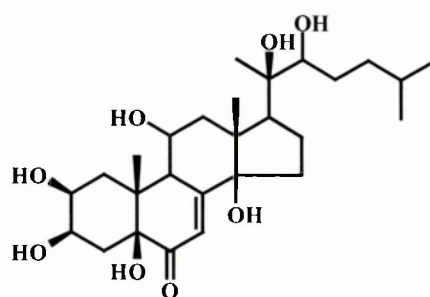
Schematic diagram describing the ecdysone-inducible mammalian expression system.

The retinoid X receptor (RXR) and a modified form of the ecdysone receptor (VgEcR), which contains the VP16 transactivation domain, are expressed in mammalian cells under the control of the rous sarcoma virus (RSV) and cytomegalovirus immediate early (CMV) promoters, respectively. In the presence of a synthetic analogue of ecdysone, such as ponasterone A (PonA) or muristerone A (MuA), the receptors form a heterodimer, the DNA binding domains (DBD) of which bind the hybrid ecdysone response elements (E/GRE). Transcription of the gene of interest (HA-S1P₄ in this instance) is activated from the minimal heat shock promoter (P_{ΔHSP}) via the VP16 transactivation domain present in the receptor heterodimer.

Synthetic ecdysone receptor hormone analogues commonly used to induce protein expression with this system are muristerone A and ponasterone A, the structures of which are shown in Figure 5.2. These compounds are steroids and possess the characteristic four-ring backbone of this class of molecule. The structure of muristerone A is identical to that of ponasterone A other than the substituents present at the 5- and 11-positions of the ring system: hydroxyl groups in muristerone A and hydrogen atoms in ponasterone A.



Ponasterone A



Muristerone A

Figure 5. 2

Structures of the ecdysone receptor hormone analogues, ponasterone A and muristerone A.

In this chapter, the ecdysone-inducible expression system was used to investigate the relationship between S1P₄ expression and agonist-independent activity. These results would indicate whether the S1P₄ constitutive activity observed in chapter 4 represented an inherent property of this receptor, or was a result of the high levels of S1P₄ expression in the cell line used for those studies.

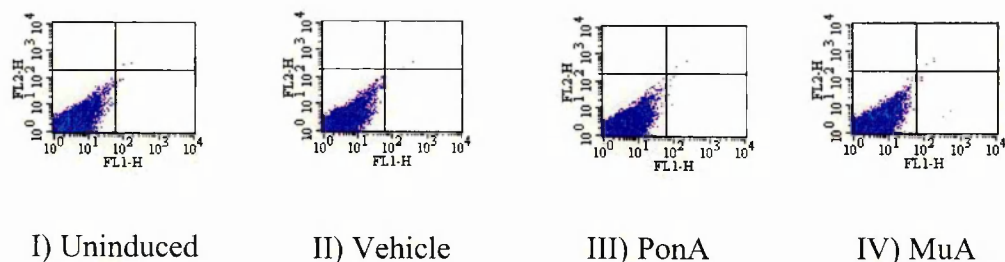
5.2 Results

5.2.1 Generation of HA-S1P₄ inducible expression stable cell line

The plasmid HA-S1P₄/pIND, which was produced in chapter 3, section 2.3 was stably transfected into CHO-EcR cells as described in chapter 2, section 3.3. This commercially available cell line had been transfected to stably express functional ecdysone receptor under selection with Zeocin. Stable transfection of these cells with the pIND plasmid containing the gene of interest followed by dual selection with Zeocin and Geneticin allowed isolation of a stable cell line in which expression of the heterodimeric ecdysone receptor was constitutive and expression of the gene of interest was under the control of the ecdysone receptor hormone analogues, ponasterone A or muristerone A.

5.2.2 Characterisation of the effect of the ecdysone receptor hormone analogues, ponasterone A and muristerone A, on cell-surface expression of HA-S1P₄

HA-S1P₄ receptor expression was measured in the stably transfected CHO-EcR cell line, which had been treated with varying concentrations of ponasterone A and muristerone A or DMSO vehicle control, for 24 hours at 37 °C. Cell-surface expression was determined via FACS analysis using an anti-HA antibody and the resultant data were expressed as fluorescence dot plots, such that staining of cell-surface HA-S1P₄ with the Fluorescein conjugated anti-HA antibody was observed as a shift to the right of the dot plot. Parental CHO-EcR cells cultured in the absence or presence of each ecdysone receptor hormone analogue, or DMSO vehicle control were analysed in this way and shown not to shift to the right under any condition (shown in Figure 5.3).

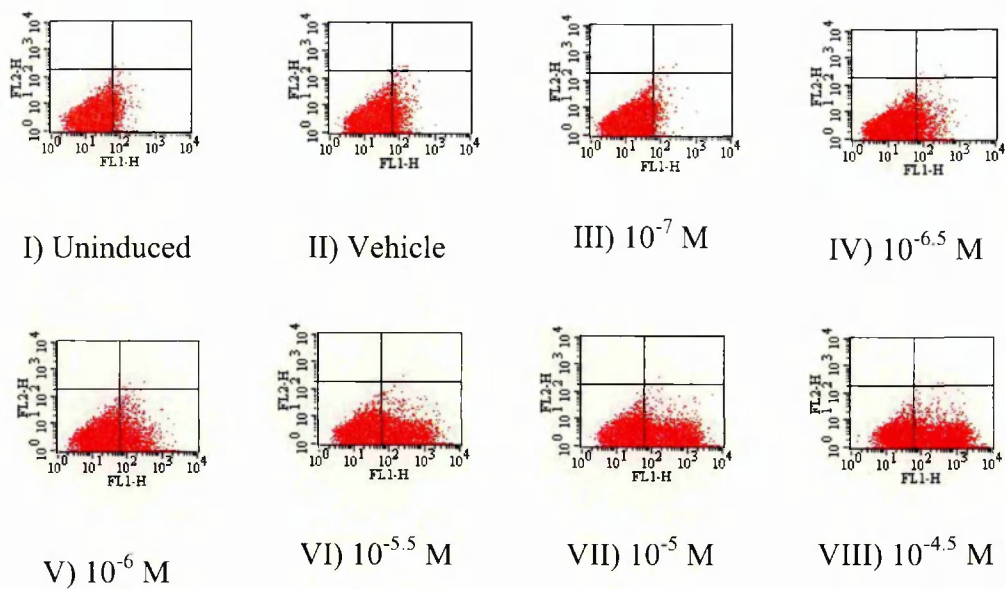
**Figure 5. 3****Dot plot analysis of parental CHO-EcR cells.**

Parental CHO-EcR cells were treated with the conditions used to induce expression in HA-S1P₄-transfected cells i.e. with DMSO vehicle, 10^{-4.5} M ponasterone A (PonA) or Muristerone (MuA), or were left untreated (uninduced) and subject to FACS analysis using a Fluorescein conjugate of the anti-HA antibody. The location of the quadrant was defined using these cells and was such that the population was present in the lower left quadrant. Data presented are from duplicate determinations of a single experiment and are representative of three independent experiments.

Quadrant analysis of the dot plots was used to quantify the percentage of cells expressing HA-S1P₄. The location of the quadrant was set using parental CHO-EcR cells, which had been stained with the anti-HA antibody (Figure 5.3) and the same quadrant location used for the analysis of HA-S1P₄ expression in the transfected CHO-EcR cell line. Comparison of dot plots from parental and HA-S1P₄-transfected cells in this manner was performed for cells treated in the same way and harvested in parallel.

Ponasterone A was titrated over the range 10^{-4.5} to 10⁻⁷ M and expression of cell-surface HA-S1P₄ determined. The dot plots (Figure 5.4 (A)) showed a small population of cells in the lower right quadrant in the uninduced and vehicle controls, which indicated that there was some constitutive expression of the receptor. The proportion of cells in this location increased with the concentration of ponasterone A and the level of expression of HA-S1P₄ was dependent on the concentration of ponasterone A used.

A



B

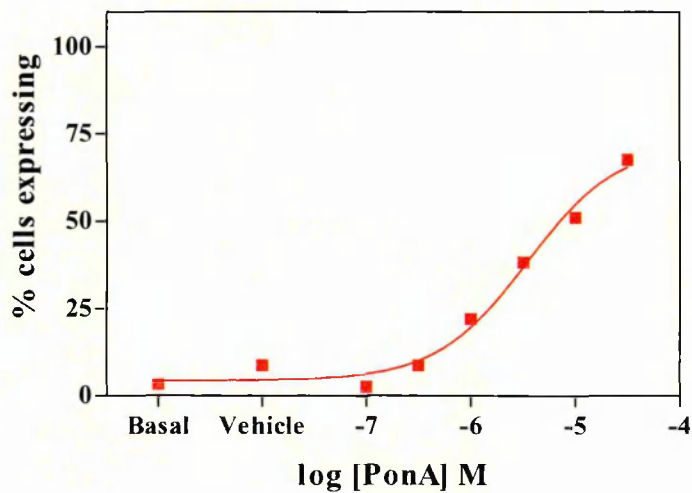


Figure 5. 4
Concentration dependent induction of cell-surface receptor expression by ponasterone A treatment of ecdysone-inducible HA-S1P₄-transfected CHO-EcR cells.
Cells were incubated for 24 hours in serum-free medium supplemented with varying concentrations of ponasterone A (PonA). Levels of cell-surface receptor expression were quantified by FACS analysis using a Fluorescein conjugate of the anti-HA antibody. Quadrant analysis of dot plots was used to determine the percentage of cells expressing HA-S1P₄ (A); the location of the quadrant was defined using CHO-EcR cells and those cells present in the lower right quadrant were classified as expressing HA-S1P₄. The resultant data are presented graphically (B). Data presented are the mean of duplicate determinations \pm SEM from a single experiment, and are representative of three such experiments performed.

When plotted as a dose-response curve (Figure 5.4 (B)), an EC_{50} value was obtained, although interpretation of this value needed caution, since a plateau of maximal receptor expression was not reached. It was not possible to use ponasterone A at concentrations in excess of $10^{-4.5}$ M since vehicle effects were observed with a vehicle control of the equivalent concentration of DMSO (not shown). At the maximal concentration of ponasterone A used ($10^{-4.5}$ M), $80 \% \pm 6 \%$ (n=3) cells were determined to be expressing HA-S1P₄.

A similar experiment was set up using muristerone A and the profile of HA-S1P₄ expression induced by this agent was found to be very similar to that observed with ponasterone A. Analysis of the dot plots (Figure 5.5 (A)) showed that a proportion of cells expressed HA-S1P₄ prior to stimulation with muristerone A, and that the number of cells present in the lower right quadrant increased with muristerone A concentration, in a dose-dependent manner (Figure 5.5 (B)). As was seen when using ponasterone A, it was not possible to use sufficiently high concentrations of muristerone A to observe a maximal plateau with this dose-response curve. At the maximal concentration of muristerone A used ($10^{-4.5}$ M), $76 \% \pm 7 \%$ (n=3) cells expressed HA-S1P₄, as determined by quadrant analysis of the FACS dot plots.

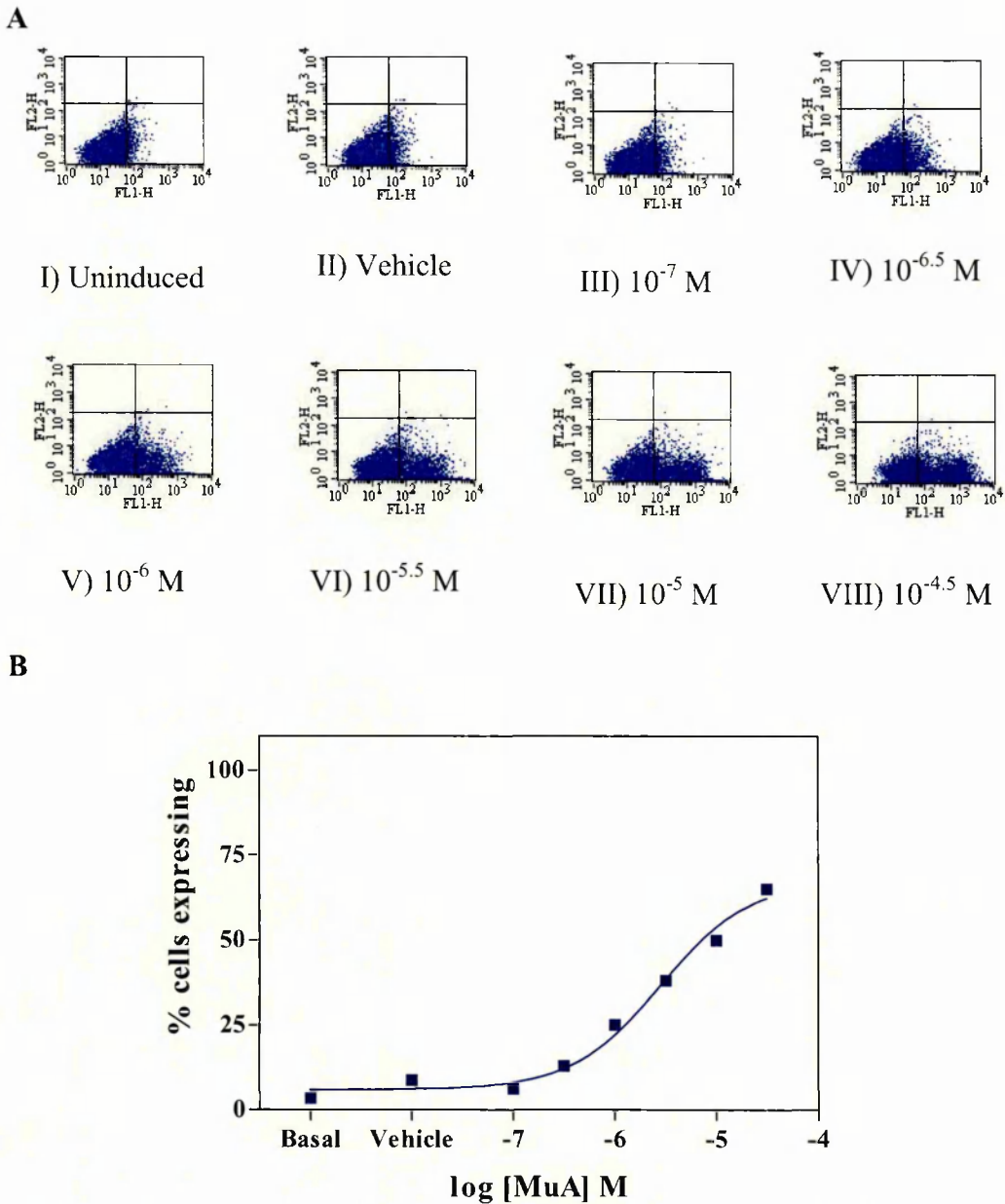


Figure 5. 5
Concentration dependent induction of cell-surface receptor expression by muristerone A treatment of ecdysone-inducible HA-S1P₄-transfected CHO-EcR cells.
Cells were incubated for 24 hours in serum-free medium supplemented with varying concentrations of muristerone A (MuA). Levels of cell-surface receptor expression were quantified by FACS analysis using a Fluorescein conjugate of the anti-HA antibody. Quadrant analysis of dot plots was used to determine the percentage of cells expressing HA-S1P₄ (A); the location of the quadrant was defined using CHO-EcR cells and those cells present in the lower right quadrant were classified as expressing HA-S1P₄. The resultant data are presented graphically (B). Data presented are the mean of duplicate determinations \pm SEM from a single experiment, and are representative of three such experiments performed.

The data obtained with each induction agent are summarised in Table 5.1.

Agent	Mean EC ₅₀ ± SEM (μM)	Maximal % cells expressing
Ponasterone A	5.44 ± 0.71	68 ± 4
Muristerone A	5.35 ± 1.29	64 ± 4

Table 5. 1

Summary of ecdysone analogue potency for induction of HA-S1P₄ expression in HA-S1P₄-transfected ecdysone-inducible CHO-EcR cells.

EC₅₀ values were calculated from dose-response experiments using the ecdysone analogues ponasterone A and muristerone A to induce cell-surface expression of the HA-S1P₄ receptor. The proportion of cells expressing the receptor was assessed using FACS analysis with a Fluorescein conjugated anti-HA antibody. The values shown above are the means of three independent determinations.

Fluorescence microscopy was used as an alternative method of showing induction of HA-S1P₄ expression as a dose-dependent response to the induction agent. Cells were cultured on microscope slides, in the presence of varying concentrations of ponasterone A, or vehicle control. Staining was performed with the nuclear stain DAPI, and a Fluorescein conjugate of the anti-HA antibody. Images of green fluorescence (due to anti-HA-Fluorescein) and blue fluorescence (due to DAPI) were viewed using a fluorescence microscope and the two images obtained for each sample were merged. These results (Figure 5.6) correlated well with the previously generated FACS data, although the fluorescence microscope was not as sensitive as the FACS method and this technique was less suitable for quantitation of the proportion of cells expressing HA-S1P₄ at a given concentration of ponasterone A. No staining with anti-HA-Fluorescein was observed when cells were cultured in vehicle control (Figure 5.6 (A)), however at the lowest concentration of ponasterone A used (10^{-6.5} M, Figure 5.6 (B)), a very small proportion of the cells had been stained with the antibody. As the concentration of ponasterone A was increased (Figure 5.6 (C))

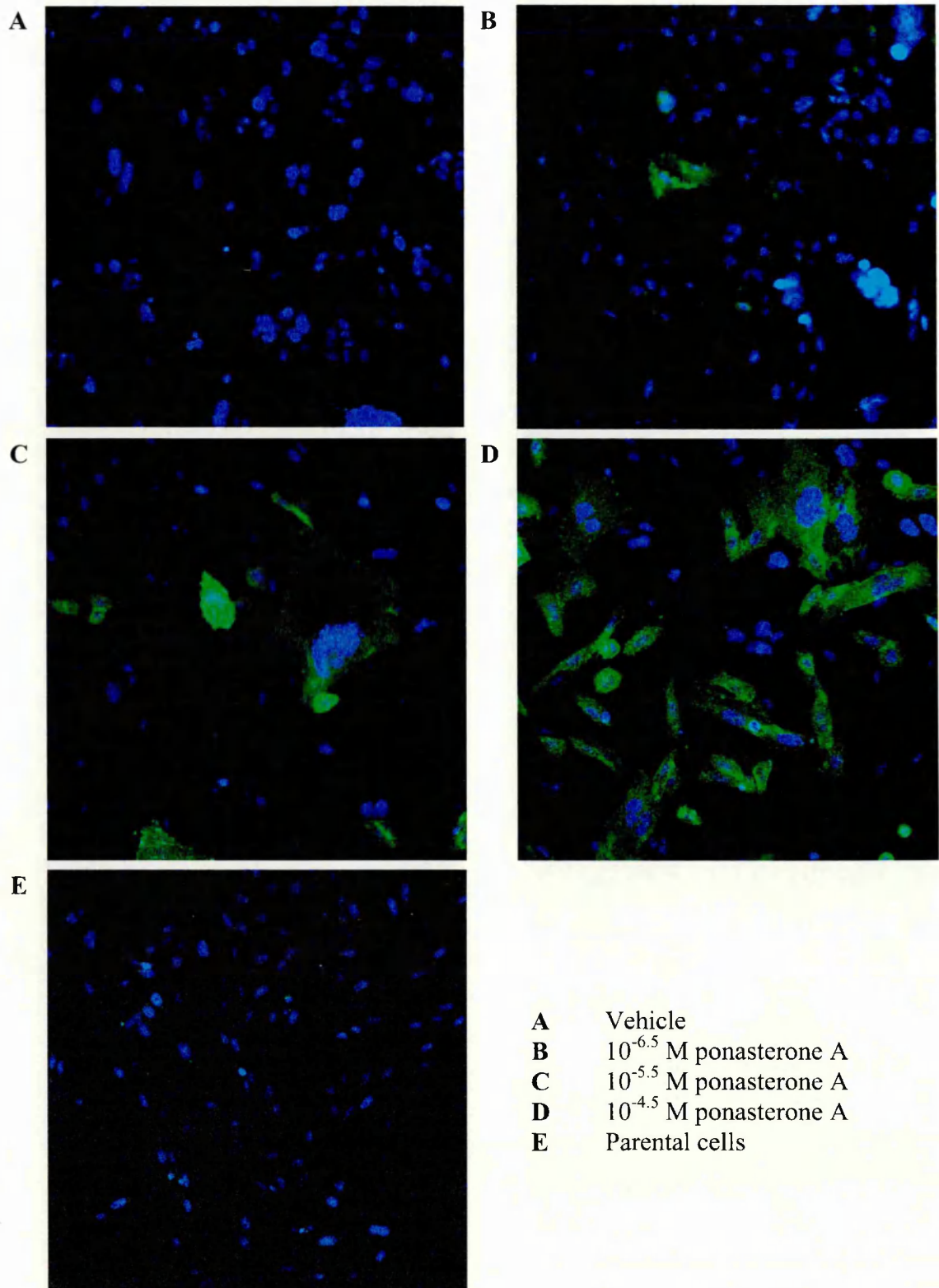


Figure 5. 6

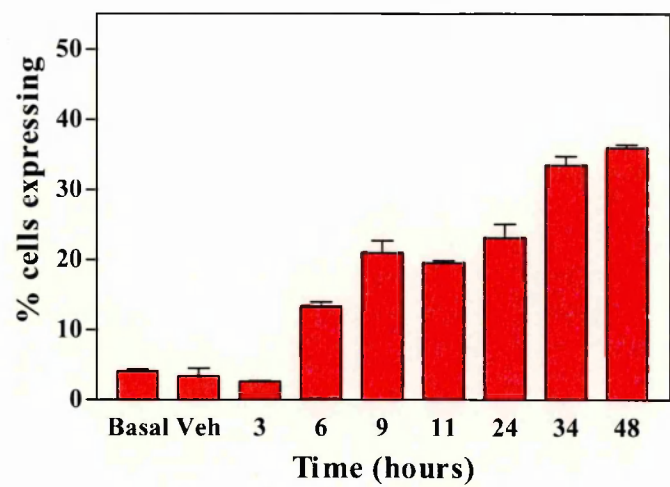
Induction of cell-surface receptor expression by ponasterone A treatment of ecdysone-inducible HA-S1P₄-transfected CHO-EcR cells detected by fluorescence microscopy.

Cells were cultured in the presence of ponasterone A or DMSO vehicle control and analysed by fluorescence microscopy (x16 magnification) using a Fluorescein conjugate of the anti-HA antibody; cell nuclei were counterstained with DAPI. Data are representative of two such experiments performed.

and (D)), the percentage of cells fluorescing green increased and some cells were seen to fluoresce more brightly than other cells. As was observed using FACS analysis, less than 100 % of cells were positive for HA-S1P₄ expression at the maximal concentration of ponasterone A used. This was in contrast to the constitutively expressing HA-S1P₄-transfected cell line described in chapter 4, where 100 % of cells stained with anti-HA-Fluorescein (data not shown). No staining was seen when untransfected cells were used (Figure 5.6 (E)), or when a Fluorescein conjugate of an isotype control antibody was used (data not shown). Staining of cell nuclei with DAPI allowed visualisation of cells when there was no green fluorescence due to binding of the anti-HA-Fluorescein antibody. Since the cells were unfixed and unpermeabilised, staining with the anti-HA antibody reflected expression of cell-surface HA-S1P₄.

The time dependency of receptor expression in the HA-S1P₄-transfected cell line was examined using a single concentration of ponasterone A or muristerone A. Quantitation of the proportion of cells expressing HA-S1P₄ was performed in the same way as described for the dose-response experiments. Exposure of cells to 10^{-5.5} M each agent over a range of incubation times from 3 hours to 48 hours resulted in a time-dependent increase of HA-S1P₄ expression (Figure 5.7). The profiles of time-dependent induction obtained with ponasterone A and muristerone A were broadly similar. Exposure to the agent for less than 3 hours did not increase cell-surface expression of HA-S1P₄, as compared to the uninduced control whilst maximal expression levels were achieved after 34-48

A



B

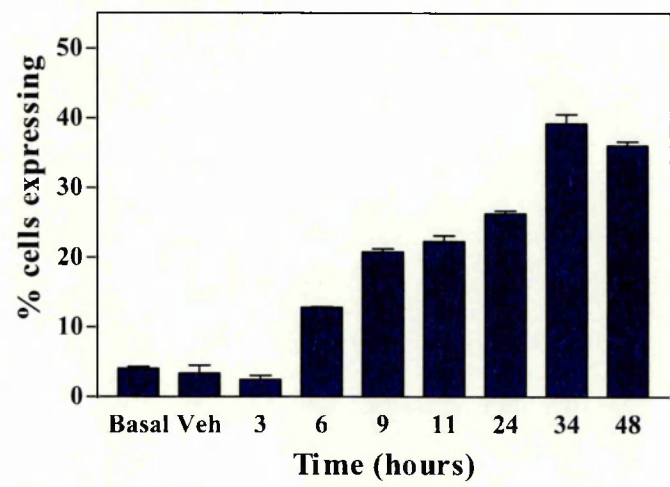


Figure 5. 7
Time-dependent induction of cell-surface receptor expression by ponasterone A and muristerone A treatment of ecdysone-inducible HA-S1P₄-transfected CHO cells.

Cells were incubated for varying times in serum-free medium supplemented with 10^{-5.5} M ponasterone A (A), 10^{-5.5} M muristerone A (B) or DMSO vehicle control (veh). Cell-surface receptor expression was quantified by FACS analysis using a Fluorescein conjugated anti-HA antibody. Quadrant analysis of dot plots was used to determine the percentage of cells expressing HA-S1P₄. The resultant data presented graphically, are the mean of duplicate determinations ± SEM from a single experiment and are representative of two independent experiments.

hours. As was observed when the concentration of induction agent was titrated, small populations of HA-S1P₄-expressing cells were detected in the uninduced

and vehicle controls. After 24 hours, approximately 25 % of cells expressed HA-S1P₄ when induced with either ponasterone A or muristerone A. This was slightly lower than the proportion of cells assessed to be expressing HA-S1P₄ at 10^{-5.5} M of either agent in the dose-response experiments and may have been due to differences in cell density at the time of induction.

Since there was no significant difference with respect to the concentration- and time-dependency of HA-S1P₄ expression induced by each induction agent, subsequent experiments were performed using ponasterone A.

Immunoblots of membranes from cells stably transfected to inducibly express HA-S1P₄ contained a polypeptide with an apparent M_r of 65 kDa, which reacted with anti-HA and anti-S1P₄ antibodies (Figure 5.8). It was possible to detect HA-S1P₄ protein in membranes prepared from cells treated with 10^{-4.5} M or 10^{-5.0} M ponasterone A, but not when lower concentrations of this induction agent were used. This presumably reflected the lower sensitivity of this technique, compared to analysis of expression by whole cells using FACS. The apparent mass of HA-S1P₄ expressed using the ecdysone-inducible system was slightly lower than that observed using the constitutively expressing HA-S1P₄ membranes (chapter 4, section 2.3). However, the apparent mass of HA-S1P₄ expressed using the ecdysone-inducible system was still greater than that predicted on the basis of amino acid sequence (43 kDa), suggesting that the small mass differences observed between expression systems were more likely due to post-translational variations, rather than proteolysis. Cross-reactivity with the anti-HA antibody confirmed the amino-terminus of the protein was intact. The

apparent molecular mass of HA-S1P₄ expressed using this system was found to be consistent over a number of clones tested (data not shown).

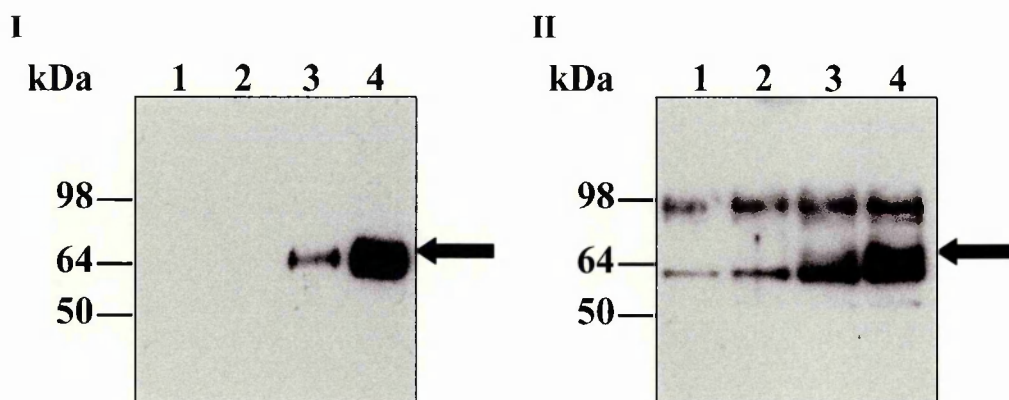


Figure 5. 8

Analysis of receptor expression in ecdysone-inducible HA-S1P₄-transfected CHO-EcR cells using Western blotting.

Membranes from untransfected CHO-EcR cells (Lane 1) and CHO-EcR cells transfected to inducibly express HA-S1P₄, and cultured in the presence of 10^{-5.5} M (Lane 2), 10^{-5.0} M (Lane 3), or 10^{-4.5} M ponasterone A (Lane 4) were analysed by Western blotting using anti-HA (panel I) or anti-S1P₄ (panel II) antibodies. Visualisation of immunoreactive proteins was achieved using chemiluminescence after incubation of the blot with appropriate HRP-conjugated secondary antibodies. The position of HA-S1P₄ is indicated by arrow.

This characterisation work enabled conditions under which to culture cells for subsequent preparation of membranes and evaluation of receptor functionality in the [³⁵S]GTPγS binding assay to be chosen.

5.2.3 The effect of cell-surface receptor expression level on HA-S1P₄ basal activity in [³⁵S]GTPγS binding assay

In an initial experiment, transfected and parental cells were seeded in complete medium and cultured for 24 hours to allow cells to attach to the tissue culture plastic. The medium was removed, cells were washed and then cultured for 24 hours in serum-free medium supplemented with a range of concentrations of ponasterone A to induce expression of HA-S1P₄. For each concentration of

ponasterone A, two flasks of cells were prepared and cultured with or without 100 ng/mL pertussis toxin. Expression of cell-surface HA-S1P₄ was confirmed using FACS analysis before membranes were prepared. Basal and S1P-stimulated signalling was measured using the [³⁵S]GTPγS binding assay. The results from this single experiment (not shown) indicated that at no concentration of ponasterone A was constitutive activity of HA-S1P₄ observed, however the addition of 10 μM S1P could stimulate [³⁵S]GTPγS binding at the highest concentration of ponasterone A used (10^{-4.5} M). The ability of S1P to activate the receptor clearly confirmed its functionality but the lack of constitutive activity was unexpected. The induction of HA-S1P₄ expression in serum-free conditions was a consequence of the experimental design and resulted in very artificial circumstances under which the receptor was expressed. A further preliminary experiment was set up where cells were seeded in complete medium supplemented with 10^{-4.5} μM ponasterone A and cultured for 24 hours before being transferred to serum-free medium containing the same concentration of ponasterone A with and without pertussis toxin. Cells were cultured for 24 hours under these conditions before being harvested. This resulted in expression of HA-S1P₄ for 48 hours, the first 24 of which were in serum-containing conditions. Comparison of the basal and S1P stimulated activities of the 48 hour-induced cells with the original membranes from cells induced with 10^{-4.5} M ponasterone A for 24 hours in serum-free conditions in the [³⁵S]GTPγS binding assay showed that induction of protein expression in serum-containing medium conferred constitutive activity and increased S1P-induced stimulation of the receptor. The following conditions were therefore chosen for the induction of cells for assay of constitutive activity:

- 1 Concentrations of ponasterone A used: $10^{-5.5}$ M, $10^{-5.0}$ M, $10^{-4.5}$ M
- 2 Cells seeded in complete medium containing appropriate concentration of ponasterone A and cultured for 24 hours
- 3 Medium removed, cells washed and cultured in serum-free medium containing appropriate concentration of ponasterone A, in the presence or absence of 100 ng/mL pertussis toxin for 24 hours. Cells harvested and membranes prepared for use in [35 S]GTP γ S binding assay.

HA-S1P₄-transfected and parental CHO-EcR cells were cultured under these conditions and the resultant membranes used to investigate HA-S1P₄ constitutive activity. Increased receptor expression caused by incubation with increased concentrations of ponasterone A resulted in increased basal activity of the membranes (Figure 5.9). Pertussis toxin was used to illustrate the extent of this constitutive activity since the level of [35 S]GTP γ S binding observed with membranes prepared from pertussis toxin-treated cells was the same regardless of the level of receptor expression.

The level of constitutive activity observed at the highest level of HA-S1P₄ expression was less than that typically observed with the constitutively expressing cell lines characterised in chapter 4. This was not entirely unexpected, since the level of receptor expression achieved with the highest concentration of ponasterone A was still lower than that of the constitutively expressing cell line.

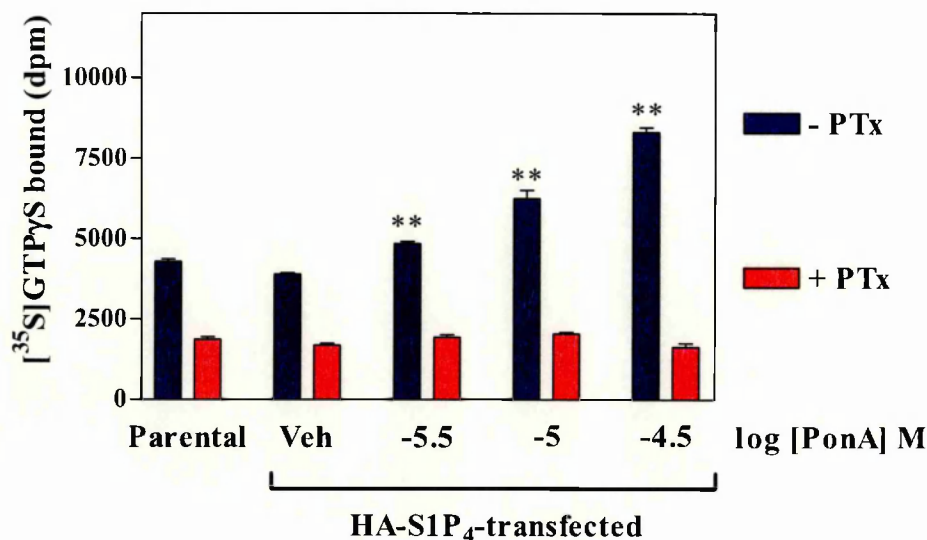


Figure 5. 9

Constitutive activity of HA-S1P₄ expressed in ecdysone-inducible CHO-EcR cells.

Membranes were prepared from parental or HA-S1P₄-transfected ecdysone-inducible CHO-EcR cells, which had been cultured in the presence of varying concentrations of ponasterone A (PonA) or vehicle (veh), and in the absence (- PTx) or presence (+ PTx) of 100 ng/mL pertussis toxin for 24 hours prior to harvest. The membranes were incubated for 5 minutes at 30 °C with [³⁵S]GTPγS and G protein α subunits immunoprecipitated after solubilisation and preclearance with non-immune serum. Data presented are the mean of three determinations ± SEM from a single experiment and are representative of two such experiments performed. Statistical significance from the response of non pertussis toxin-treated vehicle control HA-S1P₄ cells is denoted by ** (P < 0.01).

It was necessary to show that the basal activity of HA-S1P₄ was a consequence of the level of expression of this receptor and not due to the effects of residual ponasterone A present in the membrane preparations, although this seemed unlikely since cells were washed thoroughly before being harvested. Membranes from CHO-K1 cells stably expressing HA-S1P₄ (described in chapter 4) were tested in the [³⁵S]GTPγS binding assay to determine whether the maximal concentration of this agent (10^{-4.5} M) used for induction of HA-S1P₄ expression in the ecdysone-inducible cell line stimulated the receptor. Ponasterone A did

not stimulate [^{35}S]GTP γ S binding in HA-S1P $_4$ -expressing membranes (Figure 5.10). Hence the effect of cell-surface HA-S1P $_4$ expression level on basal [^{35}S]GTP γ S binding appeared to reflect genuine constitutive activity of the receptor and was not due to stimulation by residual ponasterone A.

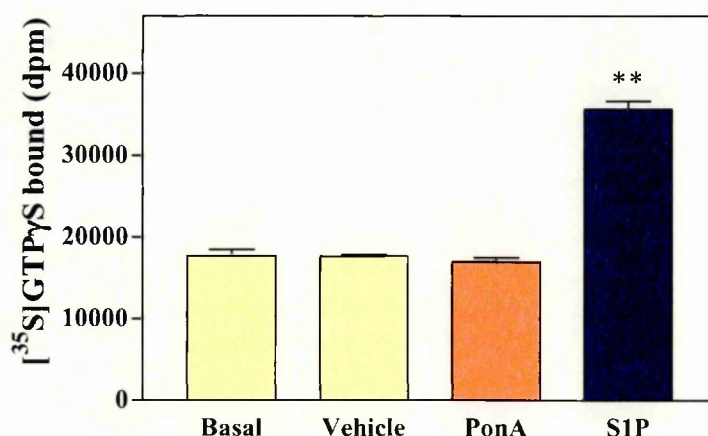


Figure 5. 10

Constitutive activity of HA-S1P $_4$ is not influenced by the presence of ponasterone A.

Membranes from HA-S1P $_4$ -transfected CHO-K1 cells were incubated for 5 minutes at 30 °C with [^{35}S]GTP γ S with 0.1 % fatty acid free BSA (basal), DMSO control (vehicle), $10^{-4.5}$ M ponasterone A (PonA) or 10^{-5} M S1P. G protein α subunits were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data presented are the mean of three determinations \pm SEM. Statistical significance from the basal response is denoted by ** ($P < 0.01$).

5.2.4 The effect of cell-surface receptor expression level on S1P-stimulated HA-S1P $_4$ activity in [^{35}S]GTP γ S binding assay

Membranes, which had been used in section 5.2.3 to characterise the effect of receptor expression on constitutive activity, were then used to investigate the relationship between the expression level of HA-S1P $_4$ and S1P-mediated increases in [^{35}S]GTP γ S binding (Figure 5.11).

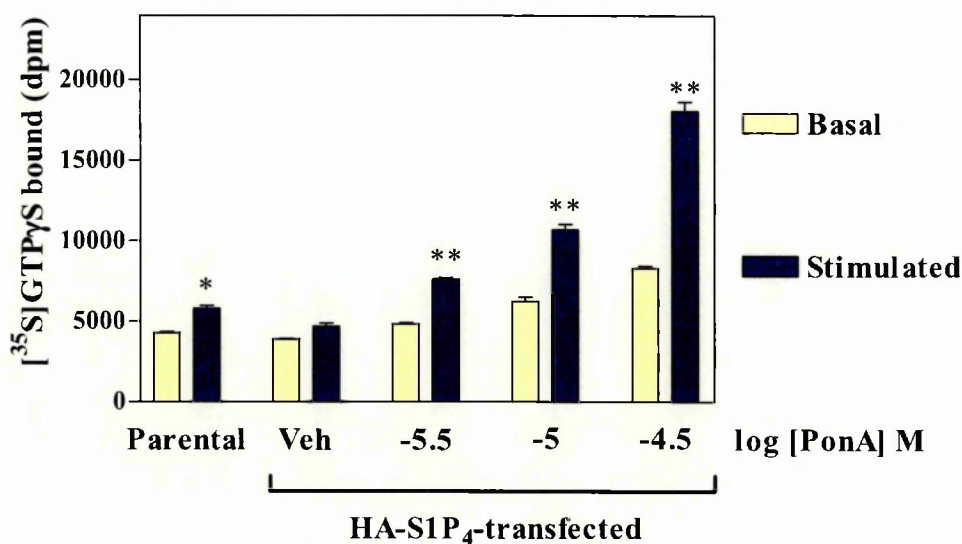


Figure 5. 11
S1P-stimulated activity of HA-S1P₄ expressed in ecdysone-inducible CHO-EcR cells.

Membranes from parental or HA-S1P₄-transfected ecdysone-inducible CHO-EcR cells, which had been cultured in the presence of varying concentrations of ponasterone A (PonA) or vehicle (veh) were incubated with [³⁵S]GTPγS for 5 minutes at 30 °C in the absence (basal) or presence (stimulated) of 10 μM S1P. G protein α subunits were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data presented are the mean of three determinations ± SEM from a single experiment and are representative of two such experiments performed. Statistical significance from the S1P-stimulated response of vehicle control HA-S1P₄ cells is denoted by * (P < 0.05) or ** (P < 0.01).

Treatment of membranes with 10 μM S1P was found to promote increased [³⁵S]GTPγS binding when compared to both the level of basal activity of those membranes, and to membranes prepared from vehicle-treated HA-S1P₄-transfected cells. The extent of [³⁵S]GTPγS binding induced by S1P increased with receptor expression, with maximal activation being obtained at the highest concentration of ponasterone A that was used. S1P was also found to cause a small stimulation of [³⁵S]GTPγS binding in untransfected CHO-EcR membranes, although this increase was not statistically significant in every experiment. Such a stimulation was not observed when parental CHO-K1 membranes were

exposed to 10 μ M S1P in experiments described in chapter 4 and may suggest that the pattern, or levels, of expression of endogenous S1P receptors in CHO-EcR cells differed slightly from that of CHO-K1 cells.

5.2.5 Correlation analysis of HA-S1P₄ constitutive and S1P-stimulated [³⁵S]GTP γ S binding activity with the level of HA-S1P₄ cell-surface expression

The relationship between the number of cells expressing HA-S1P₄, determined using the criteria described in this chapter, section 2.2, and the activity of the receptor in the [³⁵S]GTP γ S binding assay, measured in this chapter sections 2.3 and 2.4, was examined. Data were corrected by subtraction of basal dpm obtained using membranes prepared from cells which had been treated with pertussis toxin such that the resultant value solely represented the activity of HA-S1P₄. Figure 5.12 shows these data and the linear regression analysis performed on these data sets.

Both constitutive and S1P-stimulated [³⁵S]GTP γ S binding attributed to the presence of HA-S1P₄ were found to correlate strongly (Pearson's $r^2 > 0.96$) with the proportion of cells expressing the receptor; each relationship showed a good approximation to linearity ($r^2 > 0.93$) and the correlation result was statistically significant ($P < 0.001$). A summary of this analysis is shown in Table 5.2.

	Constitutive activity	S1P-stimulated activity
Linearity (r^2)	0.9346	0.9537
Correlation (Pearson's r^2)	0.9667	0.9766
Significance	$P < 0.0001$	$P < 0.0001$

Table 5. 2
Summary of statistical analyses performed on data describing the relationship between the percentage of cells expressing HA-S1P₄ and [³⁵S]GTP γ S binding attributable to the presence of this receptor.

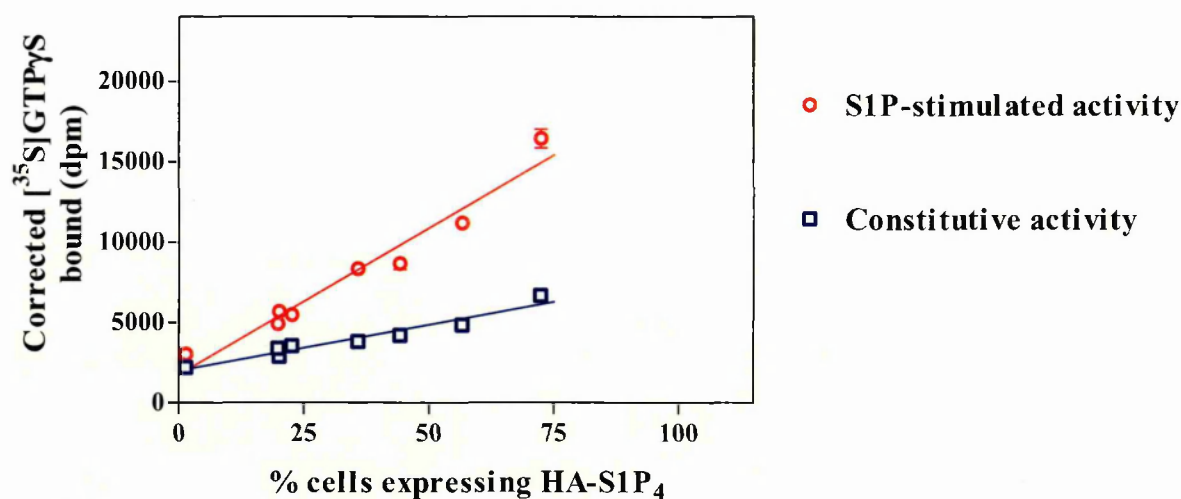


Figure 5. 12

Correlation of HA-S1P₄ constitutive- and S1P-stimulated activity from membranes prepared from ecdysone-inducible CHO-EcR cells with percentage of cells deemed to express HA-S1P₄.

Data previously obtained for basal and S1P-stimulated signalling of membranes prepared from the HA-S1P₄ ecdysone-inducible cell line, which had been cultured in the presence of varying concentrations of ponasterone A or DMSO vehicle, and the % cells expressing HA-S1P₄ determined using FACS analysis with a Fluorescein conjugated anti-HA antibody, were corrected with respect to [³⁵S]GTPγS bound for samples treated with PTx and plotted. Data plotted are from two independent experiments and are the mean of triplicate determinations ± SEM.

5.3 Discussion

The ecdysone-inducible expression system was used to further investigate the nature of basal and S1P-mediated activity of the HA-S1P₄ receptor. This system exploits the role of steroid receptors as transcription factors, the activity of which is tightly regulated by the binding of the associated steroid ligand, and uses the non-mammalian *Drosophila* ecdysone receptor as a way to achieve a tightly regulated expression system that will not suffer interference from endogenous mammalian steroids.

Since a ligand binding assay was not available for S1P₄, FACS analysis was chosen as the method of quantifying the extent of receptor expression. Flow cytometry is a sensitive technique (reviewed by Nolan *et al.*, [1999]) and the inclusion of the HA tag at the amino-terminus of the S1P₄ sequence facilitated detection of cell-surface expressed S1P₄ (shown previously in chapter 4). Quadrant analysis of FACS dot plots allowed receptor expression level to be determined and is a technique which has been used previously [Cole *et al.*, 2001]. However, this method was limited to a qualitative assessment of the percentage of cells expressing the protein of interest. Although no further information regarding the level of expression could be extracted from these data, it was straightforward to compare receptor expression caused by different concentrations of induction agent. An alternative approach might have been to measure relative receptor levels using an ELISA-based assay on fixed cells [Schoneberg *et al.*, 1995]; however this method was not tested.

Expression of cell-surface HA-S1P₄ was dependent on the concentration and the time of exposure to induction agent (ponasterone A or muristerone A). In the

absence of inducing agent, low levels of cell-surface receptor were observed. The ecdysone-inducible system has been used to express other GPCRs. In one such study low levels of the dopamine D2_{long} receptor were detected using radioligand binding assay in uninduced cells [Choi *et al.*, 2000], whilst Cole *et al.*, [2001] found that expression of mRNA and protein for the somatostatin SST₂ receptor in this system was undetectable in the absence of inducing agent.

Ponasterone A and muristerone A were found to have very similar half-maximal concentrations for dose-dependent induction of cell-surface HA-S1P₄ expression and the maximal levels of expression achieved using these agents were comparable. It was not possible to induce HA-S1P₄ expression in 100 % of cells; this is in agreement with a previous report where less than 50 % of transfected CHO-K1 cells were found to express the SST₂ receptor using a maximally effective concentration of muristerone A [Cole *et al.*, 2001]. In this study, a proportion of the SST₂-positive cells were collected using FACS and cultured before re-induction of expression. The fraction of cells expressing this receptor decreased from 100 % to approximately 65 % over a four week period. The reason for this instability is not known, but was suggested by the authors to be unrelated to cell cycle events.

Expression of HA-S1P₄ was found to reach a maximum after 48 hours exposure to ponasterone A, or after 34 hours using muristerone A. A published study with the SST₂ receptor showed maximal expression after 24 hours incubation with muristerone A [Cole *et al.*, 2001] which declined after 40 hours. Invitrogen report that the half-life of ponasterone A in culture is not known, however they observe a 30 % decrease in expression 48 hours after induction, and a 70 %

decrease after 72 hours. Muristerone A is reported to have a half-life of 24 hours. The results obtained in this chapter differ from Invitrogen's observations since HA-S1P₄ expression in response to ponasterone A was maximal after 48 hours and may suggest that temporal dependency of protein expression is related to the half-life of that protein, as well as the half-life of the induction agent in culture.

Treatment of HA-S1P₄ transfected cells with ponasterone A or muristerone A induced expression of cell-surface HA-S1P₄ in a population of those cells. At a given concentration of induction agent, FACS analysis detected a range of HA-S1P₄ expression levels. These ranged from low levels, which corresponded to a small shift into the lower right quadrant of the dot plot, to higher levels, which corresponded to a larger shift in the dot plot. Similar heterogeneity has been reported previously for this expression system [Cole *et al.*, 2001].

Fluorescence microscopy was also used to detect cell-surface expression of HA-S1P₄ and the results compared well with those generated using FACS analysis. The Fluorescein conjugated anti-HA antibody was found to give the best results with this technique. The anti-S1P₄ antibody recognises an intracellular epitope and preliminary experiments using permeabilised cells found this antibody stained less specifically than the anti-HA antibody, and also showed low levels of staining in the nuclear region of untransfected cells (data not shown).

Membranes were prepared from cells treated with different concentrations of ponasterone A, with or without pertussis toxin, then constitutive and S1P-induced activity was measured using the [³⁵S]GTPγS binding assay. In an initial experiment, protein expression was induced for 24 hours in serum-free

conditions. At none of the concentrations of ponasterone A that were used was it possible to detect S1P₄ constitutive activity and stimulation of the receptor by S1P was only observed using membranes expressing the highest level of S1P₄. It was postulated that this may have been a consequence of the use of serum-free conditions to express the receptor, so S1P₄ expression was induced using a variety of ponasterone A concentrations, in serum-containing medium for 24 hours. These cells were then cultured in serum-free medium, containing the appropriate concentration of ponasterone A in the presence and absence of pertussis toxin. Membranes prepared from these cells were shown to be constitutively active at all concentrations of ponasterone A that were used, and this activity could be further stimulated by addition of 10 μ M S1P. It is unlikely that the apparent constitutive activity of the receptor resulted from stimulation of S1P₄ by residual S1P since cells were washed thoroughly after removal of serum-containing medium prior to addition of serum-free medium for the final 24 hour culture period and were also washed thoroughly prior to harvest. It is unclear why expression using these modified conditions was necessary for the receptor to exhibit constitutive activity (and to respond to S1P when sub-maximal concentrations of ponasterone A were used) and the levels of cell-surface receptor detected using these different culture conditions were essentially identical. A key difference was the time for which protein expression was induced: 48 hours when expression was started in serum-containing medium compared with 24 hours when serum-free conditions were used. Immunoblot analysis detected no differences in apparent mass when HA-S1P₄ was expressed in serum-free conditions, compared to when expression was induced in serum-containing medium and then completed in serum-free conditions (data not

shown). A further possibility to consider is that culture of these cells in the presence of serum sustains the expression of an additional factor that is required for the detection of constitutively active S1P₄ receptor. Further investigations are necessary to determine the reason for the disparity of receptor activity between expression conditions, however it should be noted that it is more physiologically relevant to express a receptor in serum-containing conditions since S1P₄ expressed *in vivo*, for instance on a circulating T cell, would be exposed to serum continuously.

In the [³⁵S]GTPγS binding assay, basal activity increased from approximately 4000 dpm, obtained with untransfected and vehicle treated HA-S1P₄-transfected cells, to greater than 8000 dpm for cells treated with the highest concentration of induction agent. Increasing receptor expression was also shown to correlate with S1P-mediated activity. No HA-S1P₄ activity (constitutive- or agonist-stimulated) was observed when membranes prepared from vehicle treated cells were used, even though these cells expressed low levels of S1P₄. This may be a reflection of the sensitivity of the GTPγS binding assay or be due to a threshold effect whereby S1P₄ is not constitutively active until sufficient receptor is expressed to allow dimerisation or clustering; however further investigations would be needed to confirm if this were the case.

The results obtained in this chapter may be compared with those generated using the constitutively expressing HA-S1P₄-transfected cell line presented in chapter 4. Treatment of the ecdysone-inducible HA-S1P₄-transfected cell line with 10^{-4.5} M ponasterone A resulted in high levels of cell-surface expression of HA-S1P₄, although less than 100 % of cells expressed the receptor as determined

using FACS analysis. Basal, or constitutive, activity observed when $10^{-4.5}$ M ponasterone A was used to treat cells was approximately 7300 dpm, and that obtained after stimulation with 10 μ M S1P was approximately 15500 dpm. Values obtained using the constitutively expressing HA-S1P₄ cell line were approximately 10000 dpm for basal activity and approximately 19500 dpm for activity stimulated by 10 μ M S1P. Therefore, the extent of activation exhibited by the ecdysone-inducible HA-S1P₄-transfected cell line treated with the highest concentration of ponasterone A compares favourably to that obtained with the constitutively expressing cell line, despite the fact that less than 100 % of cells expressed the receptor in the inducible cell line.

Both basal and S1P-stimulated S1P₄ activity were found to show a linear relationship with the percentage of cells expressing the receptor. The activity attributable to constitutive or agonist-mediated activation of S1P₄ was calculated by subtracting the mean [³⁵S]GTP γ S binding measured using membranes prepared from cells treated with pertussis toxin from the total [³⁵S]GTP γ S binding obtained using membranes prepared from cells which had not been pertussis toxin treated. The accumulation of [³⁵S]GTP γ S in membranes prepared from pertussis toxin treated cells is not unexpected. Although ADP-ribosylation of G α_i G proteins with pertussis toxin prevents interaction of GPCR and G protein, it does not alter the intrinsic G protein functions such as guanine nucleotide exchange and GTPase activity [Gierschik, 1992]. Given that cells were categorised as expressing HA-S1P₄ if they were detected in the lower right quadrant of the FACS dot plot, and that there was no measurement of the level of receptor expression, the linearity of this relationship is intriguing. It tends to

suggest that the shape of the distribution of the extent to which cells express HA-S1P₄ is maintained and that application of ponasterone A acts to alter this population such that there is a progressive shift in the peak of this distribution, and in the size of the population in relation to the concentration of the inducer (shown as a model in Figure 5.13).

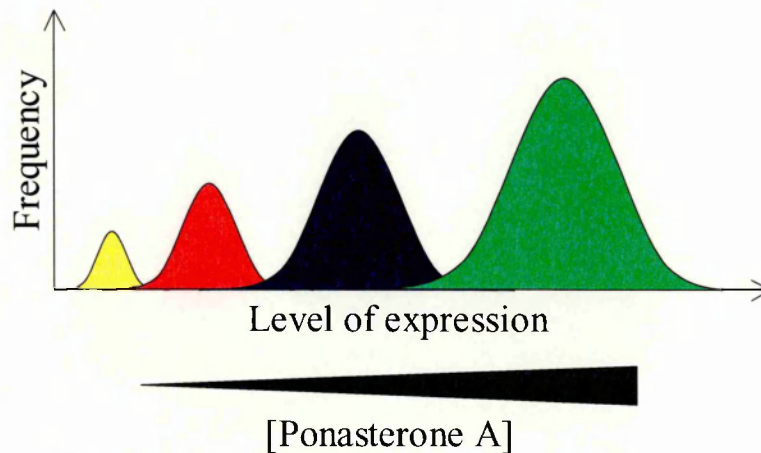


Figure 5. 13

Model to represent the effect of ponasterone A concentration on cell-surface expression of HA-S1P₄ in ecdysone-inducible CHO-EcR cells.

The population of ecdysone-inducible cells defined as expressing HA-S1P₄ using FACS analysis may be represented by a bell shaped curve and the area under the curve corresponds to the number of cells within this population. As the concentration of the induction agent ponasterone A is increased, expression of cell-surface HA-S1P₄ is increased and so the location of the population is shifted to the right. Concurrently, the area under the curve increases, reflecting increased number of cells within this population.

This investigation has shown that S1P₄ is constitutively active when expressed in CHO cells. The observed relationship between receptor expression level and constitutive activity strongly suggests that the ability of S1P₄ to signal in the absence of agonist stimulation is an inherent property of the receptor rather than merely arising due to receptor clustering or oligomerisation, which may have been a consequence of high levels of expression in a heterologous system. Constitutive activity of the S1P₄ receptor was shown to be related to expression

level and was not a consequence of stimulation of the receptor by residual ponasterone A. Treatment of membranes prepared from CHO-K1 cell transfected to constitutively express HA-S1P₄ with ponasterone A did not increase [³⁵S]GTPγS binding.

Increased levels of receptor expression are expected to lead to increased constitutive activity and indeed this has been shown for the S1P₄ receptor. The seminal study of GPCR constitutive activity [Samama *et al.*, 1993] showed the existence of a linear relationship between expression level and basal activity for both the wild type and constitutively active mutant of β₂-AR, the difference being that this function was much steeper for the mutant receptor compared with the wild type. Other studies which examined the effect of expression level on basal activity of wild type receptors also showed that increased expression resulted in enhanced constitutive activity [Cohen *et al.*, 1997; Casarosa *et al.*, 2001]. It may have been feasible to further investigate the relationship between expression level and basal activity for S1P₄ had it been possible to use a radioligand binding assay to determine absolute levels of S1P₄ expression in membranes used for the functional assay. However, since it is not known at what levels S1P₄ is expressed in a physiological setting (e.g. T cells), it is difficult to suggest whether the constitutive activity observed using this artificial system is relevant *in vivo*.

Stimulation of S1P₄ was shown using a single concentration of S1P (10 μM) and the extent of activation in response to this maximal concentration of agonist was related to expression level. It was not practical to determine whether agonist EC₅₀ values altered with increased receptor expression, however classical

receptor theory predicts that increasing receptor number would initially increase the maximal response to an agonist, without a change in potency; once that maximal response has been reached, further increases in receptor density are predicted to increase agonist potency, without affecting maximal potency. These predictions showed good agreement with experimental studies of the mGlu1 α receptor (using an IPTG inducible expression system [Hermans *et al.*, 1999]) and of the 5-HT_{2B} receptor (using sodium butyrate treatment [Jerman *et al.*, 2001]).

Other S1P receptors have been found to be constitutively active. In a landmark study using the baculovirus expression system, constitutive activation of G α_i G protein by S1P_{1/3} and G $\alpha_{12/13}$ G proteins by S1P₂ was observed [Windh *et al.*, 1999]. A recent publication describing agonist regulated and constitutive activation of specific signalling pathways by S1P₅ [Niedernberg *et al.*, 2003a] found this receptor constitutively inhibited adenylyl cyclase and activation of ERK, but that application of exogenous S1P did not further stimulate these responses. In contrast, S1P₅ mediated cell rounding via the G $\alpha_{12/13}$ pathway was found to be an intrinsic ability of the receptor, which was further modulated by S1P. These data were proposed to suggest that when a receptor is constitutively active, and is known to couple to multiple G proteins, that different effector systems may be activated to different extents.

The cannabinoid (CB) receptors are closely related to the S1P receptors and have also been reported to exhibit constitutive activity when expressed in CHO cells [Bouaboula *et al.*, 1997; Bouaboula *et al.*, 1999]. Similarly, the LPA₁ receptor was shown to activate G α_i G proteins in the absence of ligand stimulation using a baculovirus expression system [Yoshida and Ueda, 1999]. Ishii *et al.*, [2000],

also reported constitutive activity of LPA₁ and LPA₂ receptors. The recently identified second family of S1P receptors, GPR3, 6 and 12 are closely related to the Edg and CB receptor families, are constitutively active, and lack the generally conserved cysteine residue in the first extracellular loop [Uhlenbrock *et al.*, 2002]. The melanocortin (MC) receptor family comprises five members and, like the S1P and CB receptors, also lack the cysteine residue in the first extracellular loop which is generally present in family A GPCRs. This receptor family has also been shown to be constitutively active and interestingly the ligands agouti and agouti-related protein have been identified as endogenous inverse agonists at the MC receptors [Nijenhuis *et al.*, 2001].

Although the precise structural and molecular changes which correspond to receptor constitutive activity are not known, it has been suggested that conformational flexibility may contribute to this phenomenon. A study of the β_2 AR showed that a constitutively active mutant of this receptor possessed greater structural instability than the wild type receptor [Gether *et al.*, 1997]. There appears to be evolutionary pressure for most GPCRs to reside in the inactive conformation. Two striking examples are the α_{1B} AR and rhodopsin. In a landmark study of the α_{1B} AR, mutation of A²⁹³ to any other amino acid conferred a constitutively active phenotype to this receptor [Kjelsberg *et al.*, 1992]. The photoreceptor rhodopsin is maintained in an inactive conformation via covalent binding of the ligand retinal within the binding pocket and as a consequence the visual system is not subject to photobleaching that would result from constitutive activity of this receptor.

The basis for the constitutive activity observed using the S1P₄ receptor is likely to be common to the S1P, CB and MC receptor families. The unique property of these family A receptors is the lack of the generally conserved cysteine residue present in the first extracellular loop. This residue forms a disulphide bond with a second cysteine residue present in the second extracellular loop, which probably serves to constrain the transmembrane helix bundle. Since movement of transmembrane helices, particularly TM III and TM VI has been implicated in GPCR activation [Meng and Bourne, 2001], greater mobility of the helices could conceivably result in receptor constitutive activity. This notion is reinforced by observations that culture of constitutively active GPCRs with agonists and inverse agonists increases cell-surface expression of the receptor. This “ligand rescue” of constitutively active mutant receptors presumably reflects stabilisation of the receptor conformation by the binding of the ligand [Milligan *et al.*, 2002]. Conceptually, it would be interesting to examine the effect of the removal of the cysteine residue in the first extracellular loop of a typical family A receptor. In practice however, interpretation of such an experiment may be challenging since removal of this residue by mutagenesis has been reported to prevent transport of the modified receptor to the cell-surface [Ai and Liao, 2002]. An alternative approach might be to investigate whether the inclusion of a cysteine residue within the first extracellular loop attenuated S1P₄ constitutive activity. This could be achieved using a sequence alignment of family A receptors to identify the position of this cysteine residue and mutation of the appropriate residue in the S1P₄ receptor.

Expression of constitutively active mutants of GPCRs has often been reported to be lower than that of their wild type counterparts. In contrast, expression of high

levels of cell-surface S1P₄ was easy to achieve which may represent a fundamental difference between receptors that possess significant constitutive activity in their wild type state as compared to receptors which are constitutively active as a consequence of mutation.

It appears that receptor constitutive activity may be a relevant pharmacological property of certain GPCRs. The viral receptors ORF74 and US28 are constitutively active [Casarosa *et al.*, 2001] and the histamine H₃ receptor has been shown to be constitutively active *in situ* in rodent brain [Morisset *et al.*, 2000] and is thought to control histaminergic neuron activity *in vivo*. The previously discussed observation that the three receptor families which lack the cysteine residue in the first extracellular loop (S1P, CB and MC) are constitutively active lends weight to this concept. The discovery of constitutively active wild type GPCRs begs the question of whether physiological systems expressing such receptors also produce endogenous inverse agonists, which would inactivate their signalling. This has already been shown for the MC receptor family. If this was found to be the case for all constitutively active wild type GPCRs it would be an elegant symmetry of the classical system, in which endogenous agonists activate wild type receptors.

S1P₄ constitutive activity has been shown to be related to expression level and was not merely a consequence of the very high levels of expression which are commonly achieved using recombinant systems. It is unclear whether this receptor is constitutively active in its native environment, and if so, whether this property is central to the function of the S1P₄ receptor and is controlled by the actions of an as yet undiscovered inverse agonist.

Chapter 6 – Investigation into S1P₄ Ligand Preference

6.1 Introduction

It was shown in chapter 4 that S1P₄ coupled to G α_i G proteins and could be activated by the lysophospholipid S1P. Although S1P does function an agonist of this receptor, published reports have shown that S1P binds and activates S1P₄ poorly compared to its actions at other S1P receptors [Brinkmann *et al.*, 2002; Mandala *et al.*, 2002]. It has also been suggested that S1P may not be the true endogenous agonist of S1P₄ [Lynch and Im, 1999].

Significant progress has been made in the field since the start of work detailed in this thesis. Recent publications have described the actions of phosphoryl metabolites of the novel immunomodulatory compound FTY720, and its analogue AAL, as potent agonists some of the S1P receptors, including S1P₄ [Brinkmann *et al.*, 2002; Mandala *et al.*, 2002]. Additionally, the compound phytosphingosine-1-phosphate (phS1P) has been identified as a high affinity agonist for the S1P₄ receptor. A published study [Rios Candelore *et al.*, 2002] found that this compound, which is structurally similar to S1P, binds S1P₄ with approximately 50-fold higher affinity than S1P and suggested that phS1P may represent the true endogenous agonist for the S1P₄ receptor.

The first aim of this chapter was to determine the potency of these recently identified putative endogenous and synthetic S1P₄ receptor agonists using the [³⁵S]GTP γ S binding assay.

Recently, efforts have been made to understand how the S1P and LPA receptor families discriminate between their structurally similar ligands. Due to their

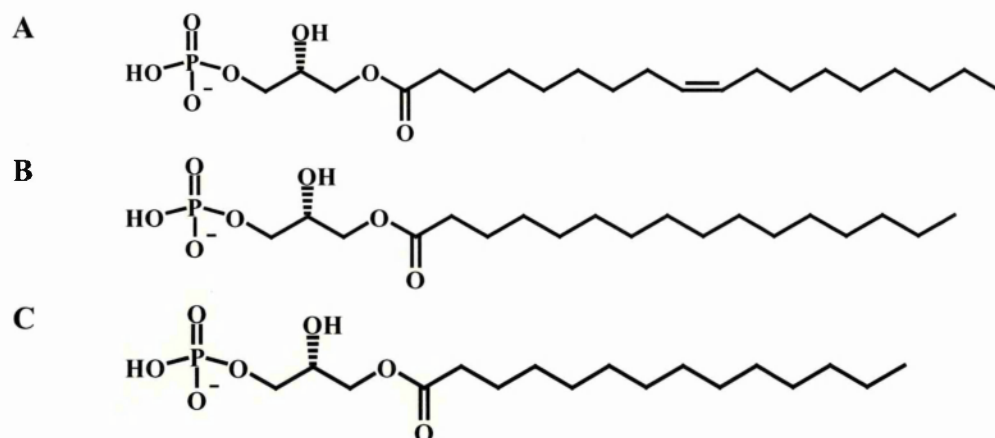
highly hydrophobic nature, direct structural studies of GPCRs are challenging, so the technique of mutagenesis is often employed in the characterisation of GPCR structure-function relationships. Alignment of the sequences of GPCRs from related families allows identification of residues which are highly conserved within receptor subfamilies, but which differ between different receptor subfamilies. This approach was adopted with the Edg family of GPCRs, where sequences of the LPA and S1P subfamilies were compared [Parrill *et al.*, 2000a; Parrill *et al.*, 2000b; Wang *et al.*, 2001; Bautista *et al.*, 2002]. A single residue in transmembrane domain III was identified as potentially influencing the ligand selectivity exhibited by each receptor subfamily. Computational modelling of S1P₁ and LPA₁ receptors, performed by Parrill and co-workers showed a probable interaction between this residue and structurally distinct groups present on the two lysophospholipid ligands, S1P and LPA. In the S1P receptor subfamily, this residue, glutamic acid (E¹²¹) was proposed to form an ion-pairing interaction with the positively charged ammonium group of S1P; in the LPA receptor subfamily, the glutamine (Q¹²⁵) residue which is present at this position was believed to hydrogen bond with a hydroxyl group on LPA [Wang *et al.*, 2001].

The second aim of the work described in this chapter was to extend this hypothesis to investigate the role of this position in the S1P₄ receptor by mutation of the naturally occurring glutamic acid to glutamine and subsequent assessment of the ability of the ligands S1P and LPA to activate this E¹²²Q S1P₄ mutant. Although the published study used RH7777 cells as a host, it was decided that the E¹²²Q S1P₄ mutant should be expressed in CHO-K1 cells. This host had been successfully used in earlier studies (described in chapter 4) and

preliminary work undertaken at the start of this thesis had been unsuccessful in detecting S1P₄-mediated increases in [³⁵S]GTPγS binding using S1P₄-transfected RH7777 cells.

It was shown in chapter 3 that CHO-K1 cells endogenously express the LPA₁ receptor, and preliminary experiments (not shown) showed that LPA stimulated [³⁵S]GTPγS binding in parental CHO-K1 cells. A fusion protein between HA-S1P₄(E¹²²Q) and the pertussis toxin-insensitive Gα_{i1}(C³⁵¹I) G protein was therefore used for these studies. Expression of this protein in CHO-K1 cells followed by treatment with pertussis toxin prior to harvest allowed elimination of any signal due to stimulation of endogenous LPA receptors. McAllister *et al.*, [2000], adopted a similar approach for studies of the LPA₁ receptor. The ability of wild type S1P₄ to couple to a tethered, pertussis toxin-insensitive Gα_i G protein was previously shown in chapter 4.

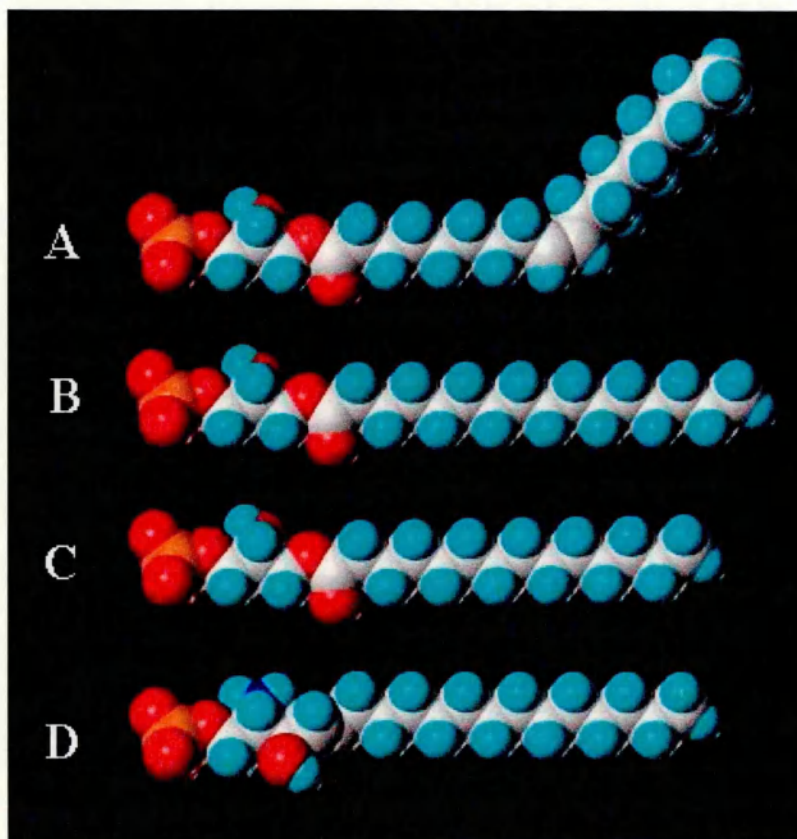
Unlike S1P, which exists as a single species *in vivo*, the term LPA actually refers to a family of molecules that take the general form 1-*o*-acyl-2-hydroxy-*sn*-glyceryl-3-phosphate. Naturally occurring forms of LPA contain acyl chains of differing lengths, with differing degrees of saturation (see Figure 6.1 for examples). Structure-activity relationship studies have examined the potency of some of the various naturally occurring forms of LPA and synthetic LPA analogues as LPA receptor agonists [Bandoh *et al.*, 1999; Bandoh *et al.*, 2000; Im *et al.*, 2000b; Heise *et al.*, 2001; Tigyi, 2001]. The length and saturation of the LPA acyl group has been shown to influence potency at isolated LPA receptors [Bandoh *et al.*, 1999; Im *et al.*, 2000b] with the general trend of decreasing potency with decreasing acyl chain length (18:1 ≥ 16:0 > 14:0).

**Figure 6. 1****Structures of three naturally occurring forms of LPA.**

- A 18:1 LPA (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate)
 B 16:0 LPA (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate)
 C 14:0 LPA (1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphate)

Comparison of space-filling models of the structures of S1P and these analogues of LPA (shown in Figure 6.2) revealed that the acyl chains of 18:1 and 16:0 LPA were longer than that of S1P. 14:0 LPA most closely resembled S1P in terms of apparent length, which suggested that 14:0 LPA might be a more effective agonist of the mutant S1P₄ receptor than 18:1 or 16:0 LPA.

The final aim of this chapter was to assess how the length of the hydrocarbon tail present on the LPA molecule affected its ability to stimulate the HA-S1P₄(E¹²²Q) receptor.

**Figure 6. 2****Models of LPA analogues and S1P.**

Space-filling models which represent the minimised extended conformation of each structure were constructed using SYBYL 6.9 software (Tripos Inc., St. Louis, MO., U.S.A.). The distance between phosphorus and terminal carbon atoms was predicted for each structure.

		Predicted distance between P and terminal C atom
A	18:1 LPA	27.0 Å
B	16:0 LPA	26.7 Å
C	14:0 LPA	24.2 Å
D	S1P	24.0 Å

6.2 Results

6.2.1 Activation of HA-S1P₄ by phS1P in [³⁵S]GTPγS binding assay

Recently, the lysophospholipid phytosphingosine-1-phosphate (phS1P) was described as a high affinity agonist for the S1P₄ receptor [Rios Candelore *et al.*, 2002]. Its potency in the [³⁵S]GTPγS binding assay was determined using membranes prepared from HA-S1P₄-transfected cells (Figure 6.3). Treatment with this agonist promoted a dose-dependent increase in [³⁵S]GTPγS binding with an EC₅₀ of 40 nM ± 8.2 nM (n=3).

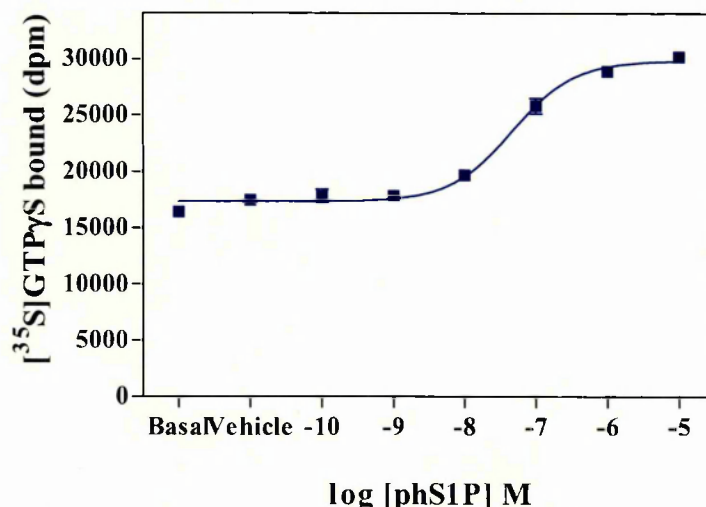


Figure 6. 3

Concentration dependent stimulation of HA-S1P₄-mediated [³⁵S]GTPγS binding by phS1P.

Membranes from CHO-K1 cells transfected with HA-S1P₄ were incubated with varying concentrations of phS1P for 5 minutes at 30 °C in the [³⁵S]GTPγS binding assay. Gα_i G proteins were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data presented are the mean of three determinations ± SEM from a single experiment and are representative of three such experiments performed.

6.2.2 Inactivity of FTY720 and AAL compounds as HA-S1P₄ receptor agonists in [³⁵S]GTPγS binding assay

The immunomodulatory compounds FTY720 and AAL have been shown to promote lymphopenia *in vivo*. This is believed to be due to the action of their phosphoryl metabolites, which have been identified as potent agonists at four of the five S1P receptors [Brinkmann *et al.*, 2002; Mandala *et al.*, 2002]. In contrast, the parent FTY720 and AAL compounds reportedly do not possess significant activity as S1P receptor agonists. These parent compounds were tested in the [³⁵S]GTPγS binding assay to determine whether they exhibited agonist activity at the S1P₄ receptor (Figure 6.4). Over the range of compound concentrations tested, neither compound stimulated a statistically significant increase in [³⁵S]GTPγS binding when compared to the basal response. Hence these compounds were considered to be inactive at the S1P₄ receptor.

6.2.3 *In vitro* phosphorylation of FTY720 and AAL compounds via incubation with rabbit blood

Phosphoryl metabolites of the compounds FTY720 and AAL (FTY720-P and AAL-P) have been reported to act as potent agonists of certain S1P receptors, including S1P₄ [Brinkmann *et al.*, 2002; Mandala *et al.*, 2002]. *In vitro* phosphorylation of these compounds may be achieved by incubation with purified sphingosine kinase 1 enzyme or with whole blood from species including rabbit, mouse and human. Parent compounds were synthesised by the Medicinal Chemistry department of Celltech R&D Ltd., and attempts made to convert each compound to the phosphate metabolite via incubation with whole rabbit blood, using a method based on that reported previously [Mandala *et al.*, 2002].

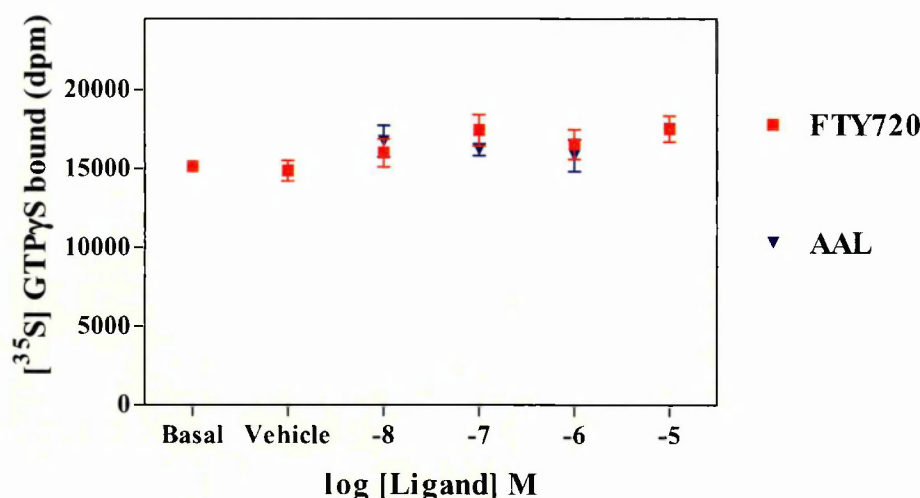


Figure 6. 4

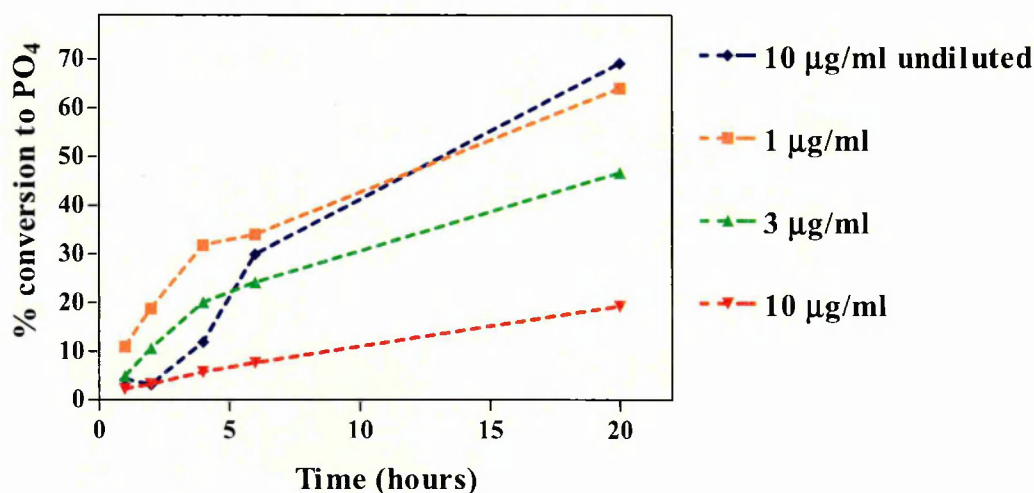
FTY720 and AAL compounds are unable to stimulate HA-S1P₄-mediated [³⁵S]GTPγS binding.

Membranes from CHO-K1 cells transfected with HA-S1P₄ were stimulated with varying concentrations of FTY720 or AAL compounds for 5 minutes at 30 °C in the [³⁵S]GTPγS binding assay. Gα_i G proteins were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data are the mean of three determinations ± SEM from a single experiment and are representative of two such experiments performed.

Incubations of varying concentrations of each compound were performed at 37 °C using undiluted whole rabbit blood, or whole rabbit blood that had been diluted 1 in 4 with supplemented RPMI medium. Samples were removed after various timepoints and the percentage conversion to phosphate derivatives determined using liquid chromatography-mass spectrometry (LC-MS). The profile obtained for FTY720 (Figure 6.5 (A)) showed time-dependent phosphorylation of this compound, with a maximal conversion of approximately 70 % achieved after overnight incubation with an initial concentration of 10 µg/ml FTY720 in undiluted blood. In contrast, the same concentration of compound incubated with diluted blood had only undergone approximately 20 % conversion after this time. The related AAL compound showed a rather different

profile of conversion to AAL-P (Figure 6.5 (B)). The maximal level of conversion was achieved using an initial concentration of 1 $\mu\text{g/ml}$ AAL and was approximately 30 % after incubation for 4 hours.

A



B

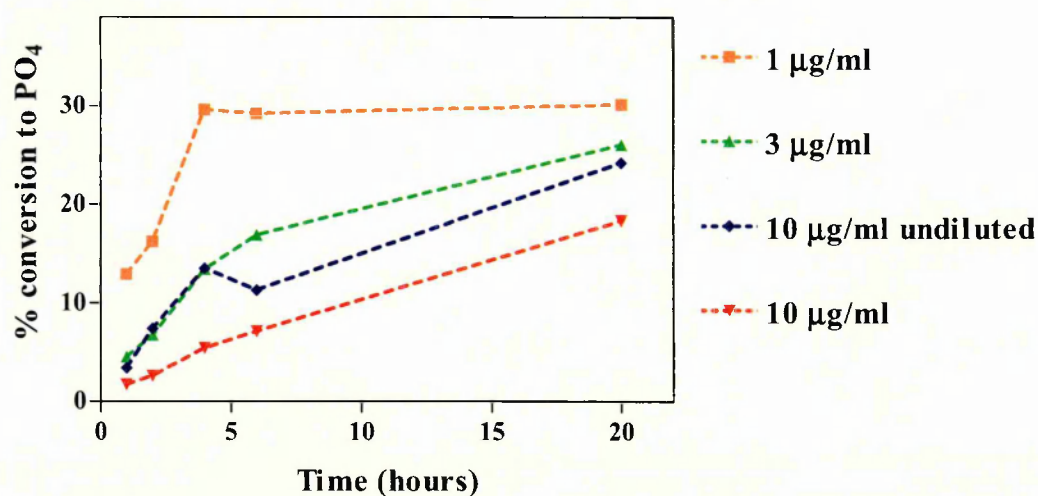


Figure 6. 5

Conversion of FTY720 and AAL compounds to phosphate metabolites via *in vitro* incubation with whole rabbit blood.

Compounds were prepared in ethanol and the concentrations shown incubated at 37 °C with whole rabbit blood that had been diluted 1 in 4 with supplemented RPMI medium, or with undiluted blood. Conversion of parent compound to the phosphoryl metabolite was monitored using liquid chromatography-mass spectrometry (LC-MS).

A FTY720

B AAL

These results allowed identification of optimal conditions to try to produce sufficient FTY720-P and AAL-P to allow isolation and purification of these phosphate metabolites. These compounds would then be tested in the [^{35}S]GTP γ S binding assay to determine their potency against the HA-S1P $_4$ receptor. Conditions were chosen using the absolute amount of phosphate produced, rather than merely the most efficient conversion in terms of percentage, so as to minimise the amount of rabbit blood required. Therefore, for FTY720, although incubation of 1 $\mu\text{g/ml}$ compound overnight with diluted blood gave the highest percentage of conversion, optimal conditions for the preparative experiment were 10 $\mu\text{g/ml}$ in diluted blood, incubated overnight. The same conditions were chosen for the preparative phosphorylation of the AAL compound.

Due to the scale of the planned preparative phosphorylation experiments, an efficient method for extraction of phosphate from the diluted blood was required. It was necessary to process the sample mix to remove proteins and to reduce the volume so that it could be injected onto a high performance liquid chromatography (HPLC) column so that the phosphate metabolite of each compound could be isolated. Attempts were made to optimise extraction of the metabolites into the organic phase of a dichloromethane (DCM) extract of the sample mix. These included testing whether an initial extraction into acetonitrile was beneficial, comparison of different ratios of DCM to sample and the effect of high and low pH on DCM extraction efficiency. Unfortunately conditions which allowed recovery of a high proportion of the phosphate metabolites from the sample mix could not be identified. This meant that only a fraction of the each phosphate metabolite could have been recovered and hence the size of

The HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I)-pIRES construct was stably transfected into CHO-K1 cells (as outlined in chapter 2, section 3.3). Western blotting was used to detect expression of this fusion protein. Membranes of HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I)-transfected cells contained a polypeptide with an apparent molecular mass of approximately 110 kDa, which reacted with anti-HA, anti-S1P₄ and SG1 antibodies (Figure 6.7 (A)). The mass of this construct corresponded to that of the wild type HA-S1P₄-Gα_{i1}(C³⁵¹I) fusion protein detected previously (chapter 4, section 2.7). Confirmation of cell-surface expression of this protein was obtained via FACS analysis using the anti-HA antibody (Figure 6.7 (B)).

6.2.5 Investigation of HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) ligand preference in [³⁵S]GTPγS binding assay

HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I)-expressing membranes were prepared from cells which had been treated with 100 ng/mL pertussis toxin for 24 hours prior to harvest. The response of the HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) receptor to the ligands S1P and 18:1 LPA was assessed using the [³⁵S]GTPγS assay. These assays were performed under the same conditions used to profile the response of the wild type HA-S1P₄-Gα_{i1}(C³⁵¹I) fusion, described in chapter 4, section 2.8. Stimulation of pertussis toxin treated HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I)-expressing membranes by S1P and 18:1 LPA was small: approximately 14 % above basal with 10 μM 18:1 LPA and approximately 23 % above basal with 10 μM S1P (Figure 6.8 (A)). Although statistically significant increases in [³⁵S]GTPγS binding were observed at the highest concentration of each agonist used (10 μM), it was not possible to calculate an EC₅₀ value for either compound. The

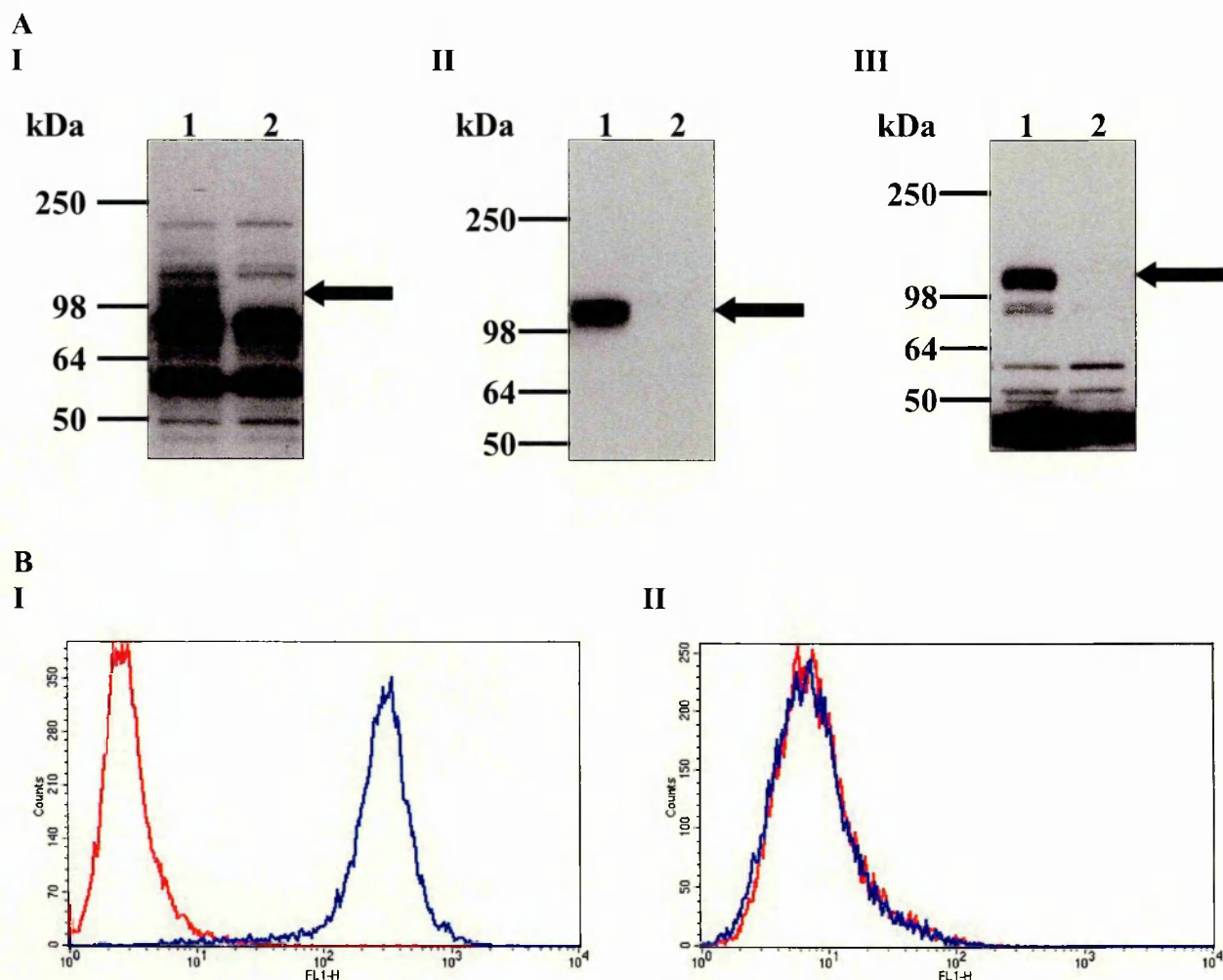


Figure 6. 7
Expression of HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) in CHO-K1 cells.
A Membranes from CHO-K1 cells stably expressing HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) (lane 1) and untransfected CHO-K1 cells (lane 2) were analysed by Western blotting using anti-S1P₄ (panel I), anti-HA (panel II) or anti-Gα_{i1} (Panel III) antibodies. Visualisation of immunoreactive proteins was achieved using chemiluminescence after incubation of the blot with appropriate HRP-conjugated secondary antibodies. The position of HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) is indicated by an arrow.
B Cell-surface expressed HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) was detected by FACS analysis using a Fluorescein conjugate of the anti-HA antibody, represented by the blue trace. Cells were also stained with an isotype matched control antibody, denoted by the red trace. Panel I shows the staining of HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I)-transfected CHO-K1 cells whilst panel II illustrates staining obtained using untransfected CHO-K1 cells. Data are presented as overlay histograms and are representative of at least five independent experiments.

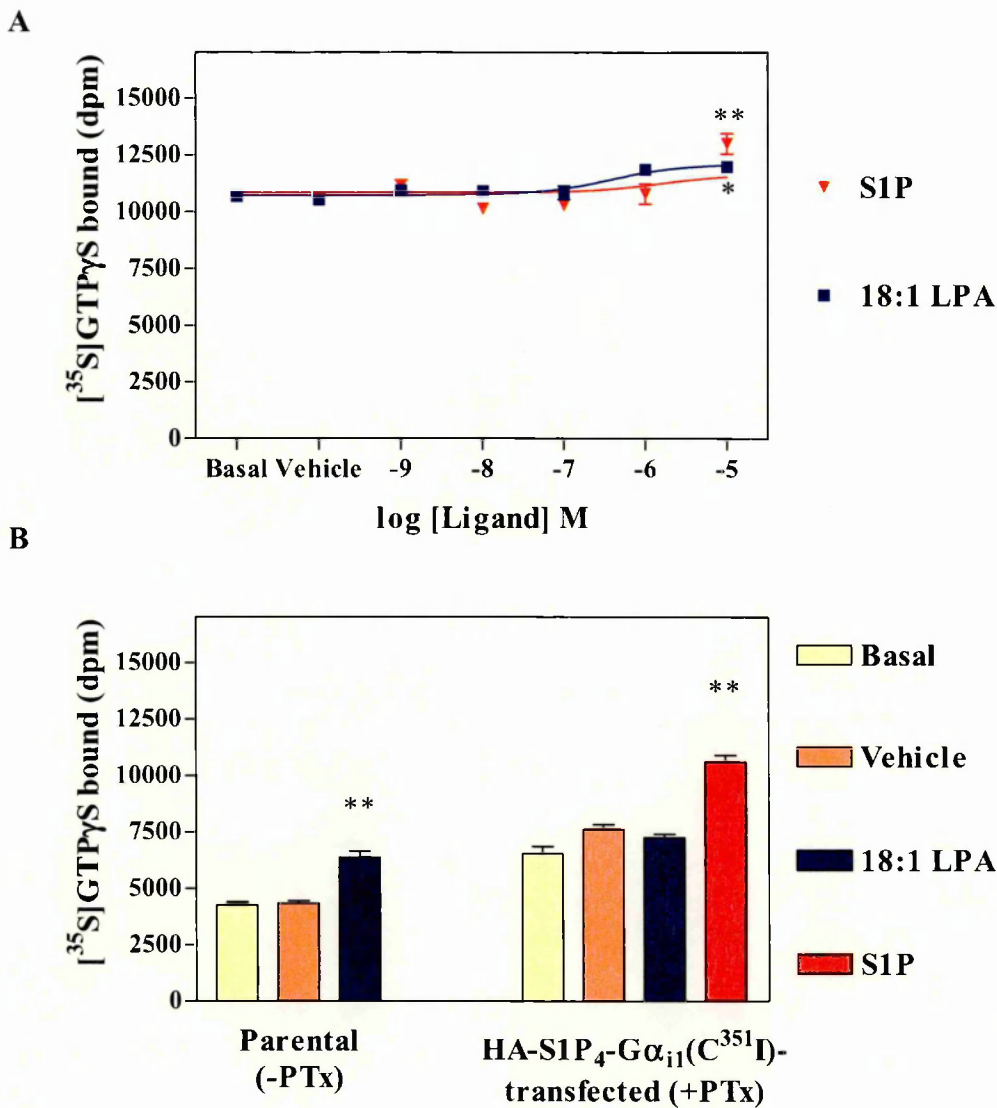


Figure 6. 8
Ligand preference of HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) in [³⁵S]GTPγS binding assay.
Membranes from CHO-K1 cells transfected to express HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) and incubated with 100 ng/mL pertussis toxin for 24 hours prior to harvest, were stimulated with various concentrations of S1P or 18:1 LPA for 30 minutes at 30 °C in the [³⁵S]GTPγS/IP binding assay (A). Membranes from parental CHO-K1 cells, or CHO-K1 cells which had been transfected to express HA-S1P₄-Gα_{i1}(C³⁵¹I) and cultured in the presence of 100 ng/mL pertussis toxin for 24 hours prior to harvest, were used as controls in this experiment and stimulated with 10 μM S1P or LPA for 30 minutes at 30 °C (B). Gα_i G proteins were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data are the mean of three determinations ± SEM from a single experiment and are representative of three such experiments performed. Statistical significance from the basal responses of each set of membranes tested is denoted by * (P < 0.05) or ** (P < 0.01).

controls for this experiment, shown in Figure 6.8 (B) demonstrated that the pertussis toxin treated wild-type HA-S1P₄-Gα_{i1}(C³⁵¹I) receptor was unable to respond to LPA, however the signalling integrity of these membranes was confirmed by stimulation with S1P. Treatment of untransfected CHO-K1 cells (which had not been pertussis toxin treated) with 10 μM 18:1 LPA increased [³⁵S]GTPγS binding and demonstrated that LPA was delivered to the assay system in a form able to activate Edg family receptors. It therefore appeared from these results that mutation of residue ¹²² to glutamine severely affected the ability of S1P₄ to respond to S1P, however this mutation did not appear to be sufficient to confer LPA-responsiveness to this receptor.

6.2.6 The effect of LPA hydrocarbon chain length on activation of HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) in [³⁵S]GTPγS binding assay

Previous studies have examined the effect of the length of the LPA acyl chain on agonist potency at receptors of the LPA family [Bandoh *et al.*, 1999; Im *et al.*, 2000b]. The ability of three variants of LPA (18:1, 16:0 and 14:0 LPA) to activate HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) was tested in the [³⁵S]GTPγS binding assay using membranes prepared from pertussis toxin-treated cells.

The preliminary experiment, shown in Figure 6.9 compared activation of HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I)-transfected membranes prepared from PTx-treated cells, by a 10 μM concentration of different forms of LPA. Both 18:1 and 16:0 LPA caused a small stimulation of [³⁵S]GTPγS binding (approximately 14 % above basal for 18:1 LPA and approximately 23 % above basal for 16:0 LPA). The level of stimulation induced by 14:0 LPA was larger (approximately 40 % above basal).

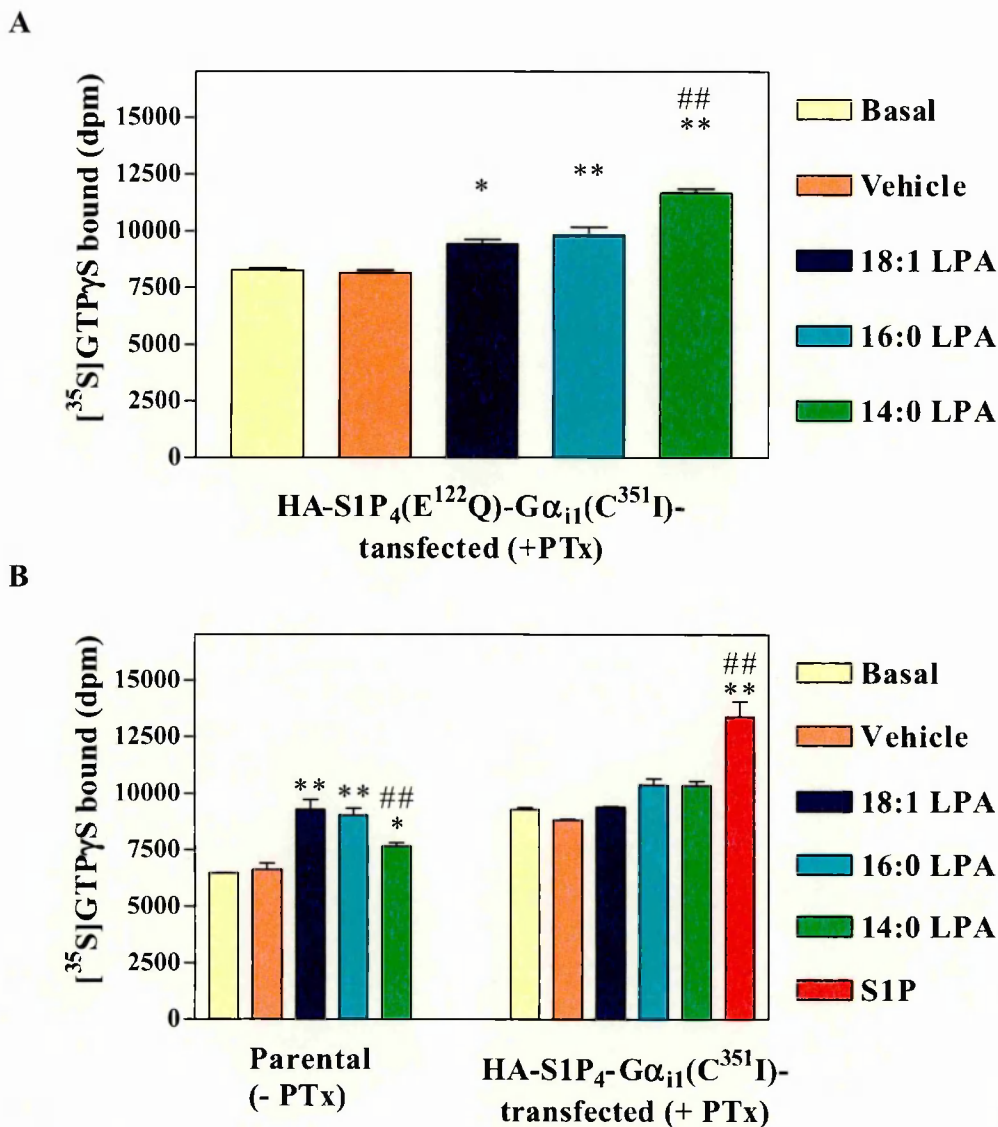


Figure 6. 9
The effect of LPA acyl chain length on stimulation of HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I).

Membranes from CHO-K1 cells transfected to express HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I), which had been cultured in the presence of 100 ng/mL pertussis toxin for 24 hours prior to harvest, were stimulated for 30 minutes at 30 °C with 10 μM of various LPA forms which contained acyl chains of different lengths in the [³⁵S]GTPγS/IP binding assay (A). Membranes from parental CHO-K1 cells, or CHO-K1 cells which had been transfected to express HA-S1P₄-Gα_{i1}(C³⁵¹I) and cultured with 100 ng/mL pertussis toxin for 24 hours prior to harvest, were used as controls (B). Gα_i G proteins were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data are the mean of three determinations ± SEM from a single experiment and are representative of two such experiments performed. Statistical significance from the basal responses of each set of membranes tested is denoted by * (P < 0.05) or ** (P < 0.01); ## denotes statistical significance from the response to 18:1 LPA (P < 0.01).

These increases were statistically significant when compared to the basal response of untreated membranes and the stimulation promoted by 14:0 LPA was statistically different to that produced by 18:1 LPA.

No form of LPA stimulated a statistically significant response in wild type PTx-treated HA-S1P₄-G α_{i1} (C³⁵¹I)-transfected membranes when compared to the basal response of these membranes, which indicated that the activity observed with the E¹²²Q S1P₄ mutant was a result of the introduction of this mutation. However, the responses induced by 16:0 and 14:0 LPA using wild type PTx-treated HA-S1P₄-G α_{i1} (C³⁵¹I)-transfected membranes were statistically different compared to the vehicle response of these membranes ($P < 0.05$). This is not thought to reflect activation of the wild type receptor by these species of LPA since the responses were not significantly different from the basal response of these membranes, but further replicates of this experiment would be needed to confirm this. Interestingly, the profile of activation of endogenous LPA receptors present on parental CHO membranes was different to that seen with HA-S1P₄(E¹²²Q)-G α_{i1} (C³⁵¹I). Untransfected CHO-K1 cells, which have been shown to express mRNA for LPA₁ (described in chapter 3) were activated by each form of LPA tested, with the rank order of maximal stimulation of 18:1 \approx 16:0 > 14:0, which was in agreement with published data [Im *et al.*, 2000b].

These results suggested that introduction of the E¹²²Q mutation in the S1P₄ receptor could confer LPA-responsiveness, and that a short form of LPA was a more effective agonist than the intermediate and longer forms, when tested at this single concentration.

6.2.7 Dose-dependent stimulation of HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) by 14:0 LPA in [³⁵S]GTPγS binding assay

The 14:0 form of LPA was tested to determine an EC₅₀ for activation of HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) (Figure 6.10). Stimulation of [³⁵S]GTPγS binding was observed at higher concentrations of ligand and was statistically significant when compared to the basal response of the membranes. The EC₅₀ value for activation of HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) was calculated to be $3.8 \pm 1.4 \mu\text{M}$. However, since a plateau of maximal stimulation was not achieved in any experiment, interpretation of this EC₅₀ value needed caution. This result clearly showed that 14:0 LPA was a weak agonist of HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) and hence confirmed the involvement of residue ¹²² in S1P₄ ligand preference. Similar results were obtained using a second CHO-K1 clone expressing this fusion protein (not shown).

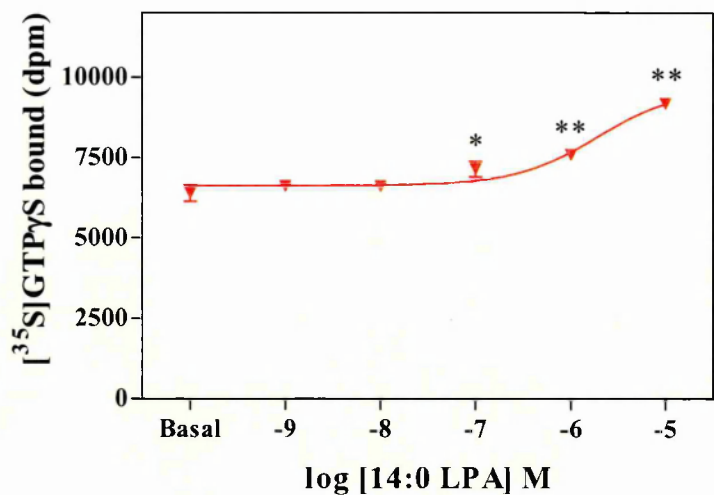


Figure 6. 10
Concentration dependent stimulation of HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I)-mediated [³⁵S]GTPγS binding by 14:0 LPA.
Membranes from CHO-K1 cells transfected with HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) which had been cultured in the presence of 100 ng/mL pertussis toxin for 24 hours prior to harvest were stimulated with varying concentrations of 14:0 LPA for 30 minutes at 30 °C in the [³⁵S]GTPγS binding assay. Gα_i G proteins were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data presented are the mean of three determinations ± SEM from a single experiment and are representative of three such experiments performed. Statistical significance from the basal response of parental cells is denoted by * (P < 0.05) or ** (P < 0.01).

6.3 Discussion

This chapter describes investigations that were undertaken to characterise the ligand preference of the S1P₄ receptor.

A recent publication described the ability of phS1P to act as a high affinity S1P₄ receptor agonist and suggested that this lysophospholipid may represent the true endogenous agonist of S1P₄ [Rios Candelore *et al.*, 2002]. This group reported that phS1P bound to S1P₄ with an apparent dissociation constant of approximately 1.6 nM, compared with the 120 nM K_d of S1P in this system. In contrast, phS1P was reported to be less potent than S1P (30.8 nM and 12.4 nM, respectively) in promoting cell medium acidification and was a partial agonist in this assay. In this chapter, the [³⁵S]GTPγS binding assay was used to show that phS1P was approximately 17-fold more potent than S1P as an agonist of S1P₄, which differs with the published study. The reason for this discrepancy is likely to be due to the different assay systems used in these determinations. The observed differences in the potency of phS1P to activate S1P₄ in these two assay systems may suggest that phS1P activates opposing pathways (whilst, presumably S1P does not), which are detected using the microphysiometer assay. The reported observation that phS1P was a partial agonist of S1P₄ was in agreement with results obtained using the [³⁵S]GTPγS binding assay where phS1P was found to have an efficacy of approximately 60 %, relative to S1P.

The potency of S1P as an agonist of S1P₄ in the cell medium acidification assay was in marked contrast to its relatively poor potency in the [³⁵S]GTPγS assay. In experiments described in chapter 4, which agreed with published studies [Brinkmann *et al.*, 2002; Mandala *et al.*, 2002], the EC₅₀ for S1P activation of

S1P₄ was approximately 355 nM whereas in the cytosensor assay its EC₅₀ was approximately 12 nM [Rios Candelore *et al.*, 2002]. There was little difference, however, in the EC₅₀ value determined for phS1P activation of S1P₄ in the published cytosensor assay (approximately 31 nM) and its potency in the [³⁵S]GTPγS assay described in this chapter (approximately 40 nM). This might suggest that the range of signalling pathways activated by S1P differs from those activated by phS1P. Results obtained using the [³⁵S]GTPγS assay indicate that each agonist is clearly able to stimulate S1P₄-mediated activation of Gα_i G proteins. Acidification of cell medium is a very general response; in contrast, the highly defined [³⁵S]GTPγS assay system offers a convenient way to directly compare the potency of different agonists, such as S1P and phS1P.

The novel immunomodulatory compounds, FTY720 and AAL were tested for their ability to activate S1P₄ and were found to be unable to stimulate [³⁵S]GTPγS binding at the highest concentration used (10 μM). This is in agreement with the reports of Brinkmann *et al.*, [2002] and Mandala *et al.*, [2002], in which the unphosphorylated FTY720 and AAL compounds exhibited no agonist activity at the S1P receptors.

The activity of phosphoryl metabolites of FTY720 and AAL compounds as potent agonists of four of the five S1P receptors is believed to reflect the *in vivo* mechanism of action of these compounds. When administered *in vivo*, FTY720 is rapidly metabolised to the phosphate, which is the predominant form of the compound in plasma [Mandala *et al.*, 2002]. *In vitro* incubation with whole blood results in phosphorylation of these compounds. This reaction is likely to be mediated by an isoform of the sphingosine kinase enzyme. The localisation

of this reaction has not been reported. Since it has been reported that endothelial cells constitutively export sphingosine kinase 1 [Ancellin *et al.*, 2002], it is possible that phosphorylation of FTY720 occurs extracellularly. Alternatively, the reaction could occur within the cytosol and the resultant phosphoryl metabolites transported out of the cell by the P-glycoprotein lipid transporter Abcb1 [Honig *et al.*, 2003]. Further work is needed to understand the precise location and kinetics of this conversion.

In this chapter, incubation of FTY720 and AAL with whole rabbit blood was used as a method for producing phosphate metabolites of these compounds. Initially, small scale incubations were performed to allow identification of optimal conditions for a larger scale incubation, which would yield sufficient phosphate derivative of each compound to allow isolation and purification. The profile of phosphorylation obtained after incubation with whole rabbit blood differed between FTY720 and AAL. FTY720 was phosphorylated relatively slowly and the maximal conversion achieved was approximately 70 %. In contrast, AAL was converted more quickly, but reached a plateau after approximately 30 % conversion. The basis for the difference in the extent to which these reactions progressed probably reflects stereoselectivity of the AAL phosphorylation reaction. The AAL compound possesses a chiral centre at the C2 atom (which is achiral in FTY720) and was produced as a racemate. It has been reported that the (R) isomer is a substrate for sphingosine kinase and shows immunomodulatory activity *in vivo*, whilst the (S) isomer is inactive *in vivo* and cannot be phosphorylated by sphingosine kinase [Brinkmann *et al.*, 2002]. The extent to which phosphorylation of the AAL compound via incubation with rabbit blood could progress was therefore limited to 50 %.

The efficiency of conversion of each compound using this *in vitro* method was low, particularly when higher concentrations of compound were used. Although purified sphingosine kinase 1 has been shown to phosphorylate FTY720 *in vitro* [Brinkmann *et al.*, 2002], the kinetics with which it does so are poor in comparison to the ability of this enzyme to phosphorylate sphingosine. Recent data have shown that the sphingosine kinase 2 isoform phosphorylates FTY720 efficiently. It may therefore have been more appropriate to attempt to generate FTY720-P and AAL-P compounds via incubation of the parent compounds with conditioned medium from endothelial cell cultures.

Large scale incubations of FTY720 and AAL with diluted rabbit blood were required to produce sufficient phosphate derivative of these compounds to allow purification and accurate quantitation of the mass and purity of the resultant products. Significant efforts were made to optimise procedures for extracting FTY720 and AAL phosphate metabolites into an organic phase prior to purification using HPLC. Although a range of conditions and solvents were tested, it was not possible to identify an efficient method. As a consequence of the low recovery of even the most efficient extraction method, the scale of incubation required to produce sufficient FTY720 and AAL phosphoryl metabolites was prohibitive. It was therefore not possible determine the potency of these compounds as agonists of the S1P₄ receptor using the [³⁵S]GTPγS binding assay. It has been reported that the phosphate derivatives of FTY720 and AAL were highly potent S1P₄ receptor agonists (EC₅₀ values in [³⁵S]GTPγS binding assay of approximately 63 nM and 4.0 nM, respectively) [Brinkmann *et al.*, 2002].

The role of residue ¹²² in S1P₄ ligand selectivity was also examined. This amino acid, which is located at the top of the third transmembrane domain, is a conserved glutamic acid in the S1P receptor family, whilst the LPA receptors contain a conserved glutamine at this position. Computational modelling of the S1P₁ receptor suggested this residue (E¹²¹ in S1P₁) was key in defining Edg family ligand selectivity due to its apparent interaction with the positively charged ammonium group present on S1P, whilst other important residues such as R¹²⁰ (present in TM III) and R²⁹² (located in TM VII) are thought to interact with structural elements common to both S1P and LPA [Wang *et al.*, 2001]. Experimental analysis of an E¹²¹Q mutant of S1P₁ suggested this model to be accurate, since the presence of this mutation conferred the ability for S1P₁ to bind, and be activated by, LPA [Wang *et al.*, 2001]. The hypothesis that this conserved residue is key in determining ligand selectivity in the S1P receptors was further tested using the S1P₄ receptor.

The HA-S1P₄(E¹²²Q) mutant, which had been constructed using PCR, and is described in chapter 3, was expressed as a fusion protein, coupled to pertussis toxin-insensitive Gα_{i1}(C³⁵¹I) G protein. This was necessary since preliminary experiments performed using the unfused mutant (not shown) were difficult to interpret due to stimulation of endogenous receptors present on CHO-K1 cells by LPA. Pertussis toxin treatment of cells expressing this fusion protein prevented activation of endogenous Gα_i G proteins by endogenous receptors thus isolating the response from the fused receptor. Expression of the HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) fusion in stably transfected CHO-K1 cells was confirmed by Western

blotting using three antibodies, which recognised different components of this protein and FACS analysis showed cell-surface expression of the receptor.

Introduction of this mutation severely affected the response to S1P. In dose-response experiments, S1P was only able to cause minimal stimulation of the HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) fusion and this occurred only at the highest concentration of ligand used. This is in agreement with published observations for activation of the equivalent S1P₁ mutant [Wang *et al.*, 2001]. However, in contrast to this group's report, the presence of this mutation in S1P₄ did not appear to confer sensitivity to activation by 18:1 LPA. Stimulation by this ligand was only apparent at the maximal concentration tested and it was not possible to calculate an EC₅₀ value from these data. The current study did not measure the ability of HA-S1P₄(E¹²²Q)-Gα_{i1}(C³⁵¹I) to bind 18:1 LPA and therefore no conclusions could be drawn as to how well the receptor could bind this ligand. It is notable, however, that S1P binds and activates the S1P₁ receptor with far higher affinity and potency than it does the S1P₄ receptor. The affinity and potency with which 18:1 LPA bound to and activated the S1P₁ mutant were approximately four-fold lower than those values for S1P at the wild type receptor, and suggest that additional interactions affect binding affinity. If the same were true for the S1P₄ mutant, it might be expected that the potency of 18:1 LPA acting at this receptor would be very low.

Interestingly, although introduction of the E¹²²Q mutation affected responses of S1P₄ to S1P, the mutant receptor was still constitutively active. This presumably reflects perturbation of S1P recognition, whilst not affecting the ability of the receptor to spontaneously adopt an active conformation. Similar observations

have been reported for the β_2 AR, where a mutation in the sixth transmembrane domain abolished agonist activation but not constitutive activity [Hannawacker *et al.*, 2002].

Investigations into the effect of the length and degree of saturation of the acyl chain of LPA have been undertaken for the LPA receptors [Bandoh *et al.*, 1999; Im *et al.*, 2000b], but no such information is available on S1P receptor which has been mutated to allow recognition of LPA. Therefore, a series of LPA forms containing different acyl chains were compared for their ability to activate the S1P₄(E¹²²Q) mutant. Based on the distance between the phosphate and terminal carbon atoms calculated using space-filling models of 18:1, 16:0, 14:0 LPA and S1P molecules, it was predicted that 14:0 LPA might be the most potent agonist of S1P₄(E¹²²Q) since it most closely resembled S1P. 18:1 and 16:0 LPA were only able to induce a small stimulation of this receptor, whereas activation in response to 14:0 LPA was larger. It had not been possible to calculate an EC₅₀ value for 18:1 LPA, and 16:0 LPA only induced a small stimulation when tested at 10 μ M so the potency of this ligand was not determined. 14:0 LPA was able to induce dose-dependent stimulation of HA-S1P₄(E¹²²Q)-G α_{i1} (C³⁵¹I) with an EC₅₀ of approximately 3.8 μ M. Whilst the LPA models used are approximate, and in reality the structures will be highly mobile and flexible, it seems possible that the longer forms of LPA tested may simply be too large to fit into the ligand binding pocket. Ligand binding data would be needed to confirm this suggestion. The results of LPA preference obtained with the S1P₄ mutant are in contrast to that shown by LPA receptors, which exhibit the general trend of 18:1 \geq 16:0 > 14:0 for potency and maximal stimulation [Im *et al.*, 2000b]. It would

be interesting to examine the affinity and potency of this series of LPA molecules with the S1P₁(E¹²¹Q) mutant to see if the same trend is exhibited. The relatively higher potency of 18:1 LPA at this receptor might make interpretation of data easier than it was for the S1P₄ mutant.

Stimulation of endogenous receptors on untransfected CHO-K1 cells by these LPA molecules followed a different trend of maximal stimulation: 18:1 \approx 16:0 > 14:0, which is in agreement to the trend observed for the LPA₁ receptor [Im *et al.*, 2000b] and is consistent with the detection of LPA₁ mRNA shown in chapter 3.

Mutation of residue ¹²² in the S1P₄ receptor from the naturally occurring glutamic acid to glutamine conferred responsiveness to 14:0 LPA whilst severely affecting responses to S1P. This observation tends to support the hypothesis that this conserved residue in the third transmembrane domain of the S1P receptors is involved in ligand recognition. This is in contrast to a recent paper describing models of several GPCRs, including S1P₄, which had been generated using novel first principle methods [Vaidehi *et al.*, 2002]. In this model of S1P₄, interactions between S1P and residues T¹²⁷ (present in TM III) and W²⁹¹ and E²⁸⁴ (present in TM VII) were observed. Interaction of E²⁸⁴ with the ammonium group of S1P appeared to control ligand selectivity since the other residues appeared to interact with the phosphate group, which is present on both LPA and S1P. It is therefore surprising that none of these residues are conserved throughout the S1P or LPA receptor families. The data presented in this chapter support the assertion that the glutamic acid residue present in the third transmembrane domain of the S1P receptors controls ligand selectivity and tend to suggest that the S1P₄ model

described by Vaidehi *et al.*, [2002] is inaccurate. However, it is also possible that residue ¹²² is not directly involved in interaction with ligand and that the introduction of the mutation has perturbed the structure of this helix such that interaction of S1P with E²⁸⁴ present in TM VII is hindered. This is one of the limitations associated with GPCR mutagenesis (discussed by Colquhoun [1998]). Further analysis, including the measurement of ligand binding affinities, of the (E¹²²Q)S1P₄ receptor is needed to further understand the role of this residue in ligand recognition. It would also be fruitful to use the mutagenesis approach to discover whether residue ²⁸⁴ is involved in S1P recognition, as proposed by the recent model of Vaidehi *et al.*, [2002]. An alternative approach that might be to generate a chimæric S1P₄ receptor that contained the third TM domain from LPA₁ to determine whether introduction of the entire α -helix from an LPA receptor could confer LPA responsiveness to an S1P receptor.

Chapter 7 – Final Discussion

7.1 Introduction

When work in this thesis was started, S1P₄ had recently been described as the orphan receptor Edg6 [Graeler *et al.*, 1998]. Phylogenetic analysis suggested that this GPCR would be activated by the lysophospholipid S1P and would couple to G proteins of the G α_i class. The first aim of this thesis was to test this prediction and attempt to measure receptor activation at the G protein level using a modified [³⁵S]GTP γ S binding assay.

S1P₄ cDNA was isolated from a PBMC library using PCR and plasmid constructs were produced to allow recombinant expression of the receptor in mammalian cell lines. One of the complications associated with the analysis of S1P receptor signalling is the presence of endogenous S1P and LPA family receptors in most cell lines. For this reason, S1P₄ was expressed both as the native receptor and as a G protein fusion. The fusion was constructed with a mutant of G α_{i1} wherein the native C³⁵¹ residue (which is the site of action of pertussis toxin) had been mutated to isoleucine, and hence was insensitive to pertussis toxin. This permitted the use of pertussis toxin to inactivate endogenous G α_i proteins and therefore eliminate any interfering responses emanating from activation of endogenous S1P receptors.

Most cell lines used for recombinant protein expression respond to S1P and LPA, which presumably reflects activation of endogenous Edg family receptors. Therefore, RT-PCR was used to determine the profile of S1P and LPA family receptors expressed in the CHO-K1 cell line that was used in these studies. Semi-redundant oligonucleotides were used to clone fragments of each of the

eight Edg family receptors from Chinese hamster genomic DNA. Sequencing of these gene fragments permitted the design of RT-PCR oligonucleotides which were specific for Chinese hamster Edg family genes. S1P_{1,2,4} and LPA₁ receptor transcripts were detected in CHO-K1 mRNA. This was the first concerted investigation into the repertoire of S1P and LPA receptors expressed in this commonly used cell line and unlike previous studies [Okamoto *et al.*, 1998; Sato *et al.*, 1999], the analysis described in chapter 3 used species-specific oligonucleotides.

In chapter 4, it was shown that S1P stimulated the S1P₄ receptor causing activation of G α_i G proteins. These observations concurred with the recently published reports of [Van Brocklyn *et al.*, 2000; Yamazaki *et al.*, 2000] which described S1P as an S1P₄ receptor agonist that promoted stimulation of pertussis toxin-sensitive pathways. In contrast to these published studies, which measured events significantly downstream of receptor activation, the results described in this chapter showed stimulation at the level of G protein activation. This was at a time when other groups reported difficulty in detecting S1P₄-mediated G protein stimulation [Im *et al.*, 2001a] and hence the results in chapter 4 were the first demonstration of S1P₄ activation at the G protein level.

Within the studies undertaken in chapter 4, it was apparent that S1P₄ exhibited a significant level of constitutive activity. It was unclear whether this was a consequence of the high levels of S1P₄ expression present in the recombinant cell lines used for these studies; therefore, further investigations into the nature of this constitutive activity were undertaken. In chapter 5, the ecdysone-inducible expression system was used to determine the relationship between

S1P₄ basal activity and receptor expression level. This study revealed a linear correlation between these variables and confirmed that the observed constitutive activity of S1P₄ represented an inherent property of the receptor and was not simply due to high levels of expression.

In chapter 6, the role of residue E¹²² was examined within the context of S1P₄ ligand preference. Each S1P receptor contains a glutamic acid residue at this position whereas a glutamine residue occupies the equivalent position in the LPA₁₋₃ receptors. This residue had previously been shown to be critical in the control of ligand selectivity for the S1P₁ receptor [Wang *et al.*, 2001] since mutation of the naturally occurring residue to glutamine attenuated S1P binding whilst conferring LPA-responsiveness to the receptor. For the S1P₄ receptor, mutation of this residue to glutamine was found to severely affect the ability of the receptor to respond to S1P. The wild type S1P₄ receptor did not respond to LPA; in contrast, dose-dependent stimulation of the (E¹²²Q) mutant by LPA was observed. A fusion protein between S1P₄(E¹²²Q) and the pertussis toxin-insensitive Gα_i(C³⁵¹I) was used in this investigation so that responses due to activation of endogenous LPA receptors could be eliminated by treatment with pertussis toxin. The results obtained suggested that residue ¹²² was important in controlling ligand selectivity for the S1P₄ receptor. Three forms of LPA were tested for their ability to stimulate the S1P₄ mutant (18:1, 16:0 and 14:0 LPA). 14:0 LPA was the most effective agonist of the S1P₄(E¹²²Q) receptor. The effectiveness of different LPA species in this experiment was proposed to have reflected the dimensions of the S1P₄ binding pocket. Space-filling models had been used to calculate the distance between the phosphorus atom of the head group and the terminal carbon atom and revealed that 14:0 LPA most closely

resembled S1P in this parameter. The other forms of LPA tested (16:0 and 18:1) were significantly longer than S1P and binding within the transmembrane helical pocket may have been compromised. This study did not assess the binding affinity of S1P and the various LPA forms to the wild type and E¹²²Q mutant S1P₄ receptors and the inclusion of this technique would have been beneficial to this investigation since these results would have illustrated whether the effectiveness of the different LPA species as agonists of the mutant S1P₄ receptor correlated with affinity. An obvious extension of this investigation would be to apply computational modelling of the S1P₄ receptor in tandem with the results described here alongside further mutational studies to gain insight into the nature of the S1P₄ binding pocket.

The use of the [³⁵S]GTPγS binding assay for the investigations presented in this thesis permitted detection of S1P₄ stimulation at the level of G protein activation. This assay may be considered to be somewhat artificial since it does not measure a “real” cellular response such as chemotaxis or activation of transcription factors. In addition, the work described here focussed solely on activation of Gα_i G proteins by this receptor and it was recently reported that S1P₄ was able to activate both G_i and G_{12/13} G proteins [Graeler *et al.*, 2003]. Clearly, the analysis of [³⁵S]GTPγS binding on Gα_i G proteins in response to S1P₄ activation is not sufficient to predict the cellular consequences of activation of this receptor. However, the use of a simple model system such as the [³⁵S]GTPγS binding assay provides insight into the first event occurring downstream of S1P₄ activation. It is appropriate to complement experiments of this type with those measuring cellular responses that may be subject to many levels of amplification, in order to build a clearer picture of the nature of S1P₄ signalling.

7.2 The *in vivo* role of S1P₄

Whilst significant progress has been made within the S1P receptor field, the *in vivo* function of S1P₄ is still unclear. Its restricted pattern of expression is suggestive of a role in immune function, however as yet no processes have been identified in which the functionality or presence of this receptor is required. The well documented effects of S1P and sphingoid mimetics (such as the phosphoryl metabolite of FTY720) on T cells appear to be S1P₁ dependent (discussed in chapter 1).

Investigations into the effect of deleting the S1P₄ gene in mice are required, although alternative approaches should also be considered given the lack of obvious phenotype observed in mice in which the related S1P₃ receptor had been disrupted [Ishii *et al.*, 2001]. The use of siRNA to silence S1P₄ in a physiologically relevant setting, for instance in a T cell line, may be useful when coupled with relevant cell based assays such as cytokine production, chemotaxis or differentiation. Alternatively, the production and analysis of “knock-in” mice expressing a non-functional form of the S1P₄ receptor might be useful. This approach is similar to the use of dominant negative enzymes and would permit direct examination of the effect of disrupting S1P₄ function (rather than simply deleting the gene) and would be more representative of the effects of an S1P₄ receptor antagonist or inverse agonist. Experiments of this nature will address the need to identify processes in which S1P₄ is key and hence will assist in elucidating the function of this receptor.

7.2.1 S1P₄ constitutive activity

The biological significance of S1P₄ constitutive activity is currently unclear, however this property is likely to be functionally relevant given the apparent evolutionary pressure on GPCRs to remain in the inactive conformation (discussed in chapter 1, section 2.4.3). S1P₄ is one of a subfamily of GPCRs, which are naturally constitutively active (S1P, LPA, CB, MC and GPR3, 6, 12 receptors). The basis for the common ability of these receptors to signal in the absence of ligand stimulation may be related to the absence of the generally conserved cysteine residue within the first extracellular loop. Although the five S1P receptors are believed to have descended from a common ancestral gene [Contos *et al.*, 2002b], there has been no suggestion that all of these constitutively active GPCRs originate from a shared ancestor. This might imply that the constitutive activity of these different receptor sub-families evolved independently and was achieved via the simplest route possible: reduction in the level of constraint of the transmembrane bundle by the loss or mutation of the cysteine residue present within the first extracellular loop. In addition, it has been reported that receptors which are caused to be constitutively active by the artificial mutation of an appropriate residue can be difficult to express [Milligan *et al.*, 2002]. This does not seem to be the case for the S1P receptors and may indicate that the basis for the constitutive activity exhibited by S1P₄ is fundamentally different from the artificial constitutive activity caused by mutation.

7.2.2 Regulation of S1P₄

The LPA_{1/2} and S1P_{2/3} receptors have been reported to mediate opposing effects [Zheng *et al.*, 2001; Takuwa, 2002], and a similar scenario may exist between S1P₄ and another S1P receptor. S1P₁ is an obvious candidate since it is expressed in many of the locations where S1P₄ is detected and its presence on T cells has been suggested to account for some of the actions of S1P on these cells [Graeler and Goetzl, 2002]. It may be that S1P₁ and S1P₄ have opposing actions which could result in the fate of a particular cell being dependent on the relative expression levels of these two receptors, as well as the efficiency of coupling to their cognate G proteins.

Another possibility to consider is the formation of heterodimers between S1P₄ and another GPCR such as S1P₁ or a chemokine receptor. The phenomenon of receptor dimerisation has been extensively studied (reviewed by Angers *et al.*, [2002]). The formation of GPCR heterodimers can be constitutive or ligand-dependent and can impart a novel pharmacology to that dimer. Heterodimer formation between the κ and δ opioid receptor subtypes was reported to produce a novel receptor with ligand binding and pharmacological properties distinct from those of the constituent receptors [Jordan and Devi, 1999]. In support of this proposal, the ability of S1P receptors to form homo- and heterodimers has been reported [Van Brocklyn *et al.*, 2002], although there is no information regarding the effect of dimerisation on S1P receptor pharmacology. Since S1P may not be the true endogenous agonist for the isolated S1P₄ receptor, formation of heterodimers with another GPCR may confer an alternative phenotype that may be related to the *in vivo* functions of this receptor. Investigations of this nature would be best pursued using a functionally relevant means of detecting

receptor dimerisation, as the traditional technique of co-immunoprecipitation may lead to spurious results. Energy transfer techniques such as BRET and FRET (reviewed by Angers *et al.*, 2000; Angers *et al.*, 2002), are less ambiguous since these methods detect the existence of GPCR oligomers on the surface of living cells.

In vivo, the resting activity of S1P₄ may be controlled by the actions of an endogenous inverse agonist countering constitutive activity. Exposure to S1P, present in normal serum and plasma may cause further stimulation of the receptor. Maximal activation could be achieved after release of S1P and hence exposure of the receptor to higher concentrations of this ligand, or by the production of the high affinity S1P₄ agonist, phS1P. It is possible that different S1P₄ agonists may promote activation of different G protein families (agonist trafficking), although there is currently no evidence to support this contention. The potential for activation of this receptor by multiple ligands, which differ in their affinity and potency, may offer increased control over receptor activation and the resultant consequences.

A further consideration is the potential for S1P₄ receptor activity to be subject to modification by accessory proteins. In recent years, it has become apparent that GPCRs may interact with other (non G protein) proteins and the effect of this interaction can alter the localisation or pharmacology of that receptor (reviewed by Brady and Limbird [2002]). This concept is exemplified by the proteins of the RAMP (receptor activity modifying protein) family, which have been shown to control the pharmacology of the calcitonin receptor-like receptor (CRLR) (reviewed by Foord and Marshall [1999]). Interaction with non G protein

proteins may alter S1P₄ signalling so that the receptor responds to different agonists, has a higher affinity for pre-existing agonists, or demonstrates enhanced or diminished constitutive activity. The identification of such proteins might aid in the elucidation of the function of this receptor.

Regulation of S1P₄ receptor expression by cell stimuli offers another level of control of the signalling of this receptor. For instance, it has been shown that expression of both S1P₁ and S1P₄ on T cells is reduced after T cell activation, and this may reflect one of the *in vivo* functions of these receptors.

7.2.3 Sphingosine-1-phosphate signalling

The enigmatic nature of S1P signalling is not yet fully understood. Whilst there appears to be a role for S1P as a second messenger, the lack of an identified intracellular target means this idea is still controversial. S1P clearly exerts some of its effects via stimulation of the S1P receptors and genetic deletion studies demonstrate a requirement for these receptors, particularly S1P₁. Studies of the effects of FTY720 on the immune system have suggested a role for this molecule (and by inference S1P) as a regulator of lymphocyte trafficking and the immune response.

Like many GPCRs, S1P receptors have been shown to internalise after ligand stimulation [Liu *et al.*, 1999; Watterson *et al.*, 2002; Graeler *et al.*, 2003] and recently the related LPA₁ receptor was found to be located on the nuclear membrane, where stimulation with LPA promoted calcium release and induction of pro-inflammatory gene expression [Gobeil, *et al.*, 2003]. It is therefore possible that S1P receptors, including S1P₄ are capable of being activated when

located intracellularly, either as a consequence of receptor internalisation or due to constitutive intracellular expression.

One thing that is clear about S1P is that it is a most unusual GPCR ligand since significant concentrations of this molecule are present in normal serum and plasma. Receptor agonists are generally tightly regulated in their production and clearance from the site of action in order that the response be transient and well defined. Both circulating and endothelial cells will be continually exposed to S1P and hence the S1P receptors on those cells may be in a state of continual stimulation, desensitisation and recycling. How this is reconciled with the constitutive activity of these receptors is unclear, however this scenario might suggest the existence of an endogenous inverse agonist. It is possible that part of the *in vivo* function of the S1P receptors is to maintain a tone via an equilibrium between receptor constitutive activity and/or exposure to S1P, and the opposing effects of any endogenous inverse agonists. Where S1P concentrations are elevated, for instance at sites of injury and inflammation, this equilibrium could be overcome and complete activation of the relevant receptors may be achieved. Similar effects might be achieved by spatial and temporal control of inverse agonist production.

7.3 Summary

Many questions have arisen from the work presented in this thesis, not least the uncertainty about the *in vivo* role of the S1P₄ receptor, the identity of its endogenous ligand(s) and functional relevance of S1P₄ constitutive activity.

Over the past 10 years, the field of lysophospholipid related research has grown rapidly and there is now abundant information regarding the signalling pathways activated by the Edg family receptors. Some of this information has been produced using artificial *in vitro* systems and some has arisen from studies of more physiologically relevant studies, including the analysis of S1P and LPA receptor-null mice. Several areas await further investigation; of particular interest will be the analysis of S1P₄ receptor function in complex tissues during normal physiological and pathophysiological situations. This is likely to be achieved via the use of genetic manipulations, possibly using conditional knockout technology. The development of blocking or activating anti-S1P₄ receptor antibodies will also be interesting and should offer an alternative, highly selective route for discovering the consequences of activation or antagonism of this receptor. The identification of selective agonists and antagonists/inverse agonists which discriminate between different S1P receptor subtypes is a limiting step in the elucidation of the physiological, and possible pathophysiological roles of the S1P receptors in immune system regulation.

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